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(54) Title: CLONING AND CHARACTERIZATION OF VIRAL IAP ASSOCIATED FACTOR (VIAF) IN SEVERAL ORGANISMS

(57) Abstract: DNA and proteins sequences are disclosed for several VIAF homologs. The VIAF sequence modulates the anti-apoptotic and signaling properties of IAP family members. The disclosure also includes specific binding agents (such as antibodies) that recognize VIAF, methods of decreasing apoptosis by increasing VIAF expression, methods of increasing apoptosis by decreasing VIAF expression, methods of treating disease caused by mutations, therapeutic compositions that include VIAF, recombinant DNA molecules, probes, and transformed cells that incorporate the DNA sequence to express VIAF. The disclosure also includes methods of diagnosis and treatment of diseases caused by an increased expression of VIAF, such as neurodegenerative diseases, and methods of treatment of diseases caused by unwanted apoptosis, such as occurs in autoimmune diseases and neurodegenerative disorders. The disclosure also includes methods of diagnosis and treatment of diseases caused by an underexpression of VIAF, such as cancer.

CLONING AND CHARACTERIZATION OF VIRAL IAP ASSOCIATED FACTOR (VIAF) IN SEVERAL ORGANISMS

FIELD

This disclosure relates to nucleic acid and amino acid sequences corresponding to VIAF genes in several species. The sequences are useful for modulating the anti-apoptotic and signaling properties of the IAP family.

BACKGROUND

The process of apoptosis, or programmed cell death, can be utilized to eliminate unwanted cells (Kerr et al., 1972, *Br. J. Cancer* 4:239-57). This can occur during embryogenesis, turnover of senescent cells or metamorphosis. It also represents a very efficient defense mechanism against pathogens, such as viruses, by allowing the host organism to eliminate infected cells. However, unwanted apoptosis can also occur, for example in pathological conditions associated with autoimmunity, AIDS, and retinal degeneration.

In response to this defense mechanism, viruses such as herpesviruses, poxviruses and insect baculoviruses, have developed counter-strategies to escape or retard the apoptosis triggered by the host organism (Henderson et al, 1993, *Proc. Natl. Acad. Sci. U S A.* 90:8479-83; Cheng et al.,1997, *Science* 278:1966-8). An example of such a viral mechanism is the expression of proteins that inhibit or regulate apoptosis. Several viral proteins that inhibit apoptosis have been identified (Eiben and Duckett, 1998, *Results Probl. Cell. Differ.* 24:91-104). One group of inhibitory proteins, such as the cowpox serpin CrmA or the baculoviral p35 gene product, act as pseudosubstrate inhibitors of one or more caspases, the known mediators of apoptosis. While CrmA inhibits only caspase-1 and caspase-8, p35 is a more broad-spectrum caspase inhibitor. These viral gene products block proteolytically active caspases by remaining tightly bound to the active site following their proteolysis.

In contrast, other baculoviruses such as *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpMPNPV) or *Cydia pomonella* granulosis virus (CpGV) do not express p35. Instead, they express the family of inhibitors of apoptosis proteins (IAP) (Clem and Miller, 1994, *J. Virol.* 67:3730-8). The IAPs have been implicated in a variety of essential cellular processes in addition to apoptosis, including signal transduction, cell cycle regulation, control of gene transcription and ubiquitination (Rothe *et al.*, 1995, *Cell* 83:1243-52; Chu *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:10057-62; Sanna *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95:6015-20). Expresson of Op-IAP in mammalian cells has been shown to confer protection from a variety of apoptotic stimuli including Fas/Apo-1/CD95 (Duckett *et al.* 1996, *EMBO J.* 15:2685-94; Hawkins *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93:13786-90; Hawkins *et al.*, 1998, *Cell. Death Differ.* 5:569-76). In insect cells IAPs have been shown to bind to the apoptotic inducers Reaper, Hid, Grim and Doom, suggesting that their protective effects may be mediated through multiple targets (Harvey *et al.*,

1996, *Mol. Cell. Biol.* 17:2835-43; Manji *et al.*, 1997, *J. Virol.* 71: 4509-16; Vucic *et al.* 1998, *Mol. Cell. Biol.* 18:3300-9). However, in mammals no analogous proteins have been identified.

The structure of the baculoviral IAPs is characterized by two types of sequence motifs. The C-terminus features a zinc binding domain known as a RING-finger, and the N-terminus exhibits one to three approximately 65 amino acid long Cys/His-rich sequences termed baculovirus IAP repeat (BIR). Both the RING finger and BIR repeats have been shown to be essential for preventing apoptosis. It has also been shown that the human homologue hILP/XIAP is a potent inhibitor of the effector caspase-3 but does not inhibit proteolytically active caspase-1,-6 or -8. This inhibition is mediated by the BIR motifs of the protein whereas the RING finger was not necessary for this interaction. Two other cellular IAPs, c-IAP1 and c-IAP2, are components of the type 2 tumor necrosis factor receptor cytoplasmic complex and shown to be required to suppress TNF-α induced apoptosis.

SUMMARY

Although the mechanism by which mammalian IAPs regulate apoptosis has been investigated, the mechanism of their action remains unknown. To gain an understanding of the mechanism used by IAPs to antagonize apoptosis, the identification of proteins which interact with the IAPs would be useful.

Herein disclosed is a novel protein, viral IAP-associated factor (VIAF), that is evolutionary conserved across several species and interacts directly with baculoviral OpIAP as well as mammalian XIAP, c-IAP1 and c-IAP2. VIAF modulates the anti-apoptotic and signaling properties of the IAP family. For example, VIAF inhibits Bax- and Fas-induced apoptosis and co-expression of VIAF with suboptimal quantities of XIAP confers almost complete protection. In certain disclosed embodiments, VIAF and XIAP synergistically stimulates c-Jun N-terminal kinase activity.

The cDNA and protein sequence of VIAF, for several different organisms including human, mouse, zebrafish, and *Drosophila*, are also disclosed.

Disclosed herein is a purified protein having VIAF biological activity, which modulates the anti-apoptotic and signaling properties of the IAP family. In some disclosed embodiments, the VIAF protein has the amino acid sequence shown in either SEQ ID NOS 2, 4, 6, or 8 or amino acid sequences that differ from those specified in SEQ ID NOS 2, 4, 6, or 8 by one or more conservative amino acid substitutions, or amino acid sequences having at least 80% sequence identity to those sequences, for example sequences that are at least 85%, 90%, 95% or even 98% or 99% identical. Other embodiments include a VIAF protein containing no more than about 240 amino acid residues, or in other embodiments, no more than about 250, 260, or 270 residues. In other embodiments, the amino acid sequence contains at least 10, 15, 20, or 25 contiguous amino acid residues of SEQ ID NOS 2, 4, 6, or 8. Also included is an isolated nucleic acid molecule

encoding a biologically active VIAF protein, particularly such molecules that include a promoter sequence operably linked to the nucleic acid molecule for expression of the VIAF protein, as well as transgenic cells containing these molecules. In addition to such variants that retain biological activity of the VIAF protein, fragments of the sequences that have or retain such activity may be used. Such fragments may, for example, include at least 50%, 75%, 90% or 95% of the amino acid residues of the native peptide sequence.

Also disclosed are animal proteins having VIAF biological activity. In particular embodiments, the animal is a higher eukaryote, for example a mammal. In yet other embodiments, the mammal is a mouse or human.

In particular embodiments, the isolated nucleic acid molecule includes at least 21 contiguous nucleotides of a sequence selected from SEQ ID NOS 3 or 7 or its complementary strand. In other embodiments, the isolated nucleic acid molecule includes at least 22 contiguous nucleotides of a sequence selected from SEQ ID NO 5 or its complementary strand. Alternatively, the isolated nucleic acid molecule includes at least 30 or 50 contiguous nucleotides of SEQ ID NOS 3, 5, or 7, or a nucleic acid molecule that is at least 80% homologous to SEQ ID NOS 1, 3, 5, or 7, and encodes a protein having VIAF biological activity. Alternatively, the nucleic acid molecule has a sequence which hybridizes under stringent conditions to the sequences defined in SEQ ID NOS 1, 3, 5, or 7, or which has the full length sequence of SEQ ID NOS 1, 3, 5, or 7 or its complementary strand. In other embodiments, the nucleic acid molecule has a sequence which hybridizes under conditions of at least 75% or 90% stringency to the sequences defined in SEQ ID NO NOS 1, 3, 5, or 7, or which has the full length sequence of SEQ ID NOS 1, 3, 5, or 7 or its complementary strand. In yet another embodiment, the nucleic acid molecule has a sequence depicted as bases 62-781 of SEQ ID NO 1, and encodes a protein having VIAF biological activity, for example the amino acid sequence shown in SEQ ID NO 2.

Another embodiment includes an isolated nucleic acid molecule which includes at least 370 contiguous nucleotides of a nucleic acid sequence shown in SEQ ID NO 1, or its complementary strand, or 20, 30, 50 or more contiguous nucleotides from nucleotides 62-334 of SEQ ID NO 1, or its complementary strand. The 20, 30 or 50 contiguous nucleotides can alternatively be taken from nucleotides 1-334 or 704-781, or 62-334 or 703-781, of SEQ ID NO 1, or its complementary strand.

Another embodiment includes isolated nucleic acid molecules (such as oligonucleotides) which are capable of specifically hybridizing to a VIAF gene, for example a nucleic acid molecule having at least 25 consecutive nucleotides of the sequences shown in SEQ ID NOS 3, 5, or 7. Alternatively, the nucleic acid molecules have at least 21, 30, or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 3; at least 22, 30 or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 5; or at least 21, 30 or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 7. In yet another embodiment, antisense oligonucleotides are disclosed which

hybridize to RNA or a plus strand of the nucleic acids disclosed herein and inhibits VIAF biological activity are provided.

Also disclosed herein are recombinant vectors that include any of the nucleic acid molecules, and transgenic hosts into which the recombinant vector is incorporated. Also disclosed are the purified peptides encoded by any of these nucleic acid molecules, such as proteins (including fusion proteins) having VIAF biological activity which can be used to modulate the anti-apoptotic and signaling properties of the IAP family. In particular embodiments, the peptide has an amino acid sequence shown in SEQ ID NO 2, 4, 6, or 8, or variants or fragments thereof.

Also disclosed are specific binding agents capable of specifically binding to a VIAF protein, for example polyclonal antibodies, monoclonal antibodies, and fragments of monoclonal antibodies that specifically bind to the VIAF protein. Such specific binding agents can be used in assays for quantitating amounts of purified VIAF, for example to diagnose diseases associated with abnormal VIAF expression.

Other embodiments include a composition having a therapeutically effective amount of a protein with VIAF biological activity, in combination with a pharmaceutically acceptable carrier, for treating conditions in which VIAF activity is impaired or lost. In other embodiments (examples), the composition further includes one or more other anti-apoptotic compounds. The protein having VIAF biological activity contained within such compositions includes any VIAF protein or peptide disclosed herein, including fragments and variants. In other examples, the composition can include a therapeutically effective amount of a specific binding agent described above and a pharmaceutically acceptable carrier. In yet other examples, the composition can include a therapeutically effective amount of antisense oligonucleotides described above and a pharmaceutically acceptable carrier.

The disclosed compositions can be used to decrease apoptosis. In other embodiments, the compositions disclosed herein can be used to inhibit Bax- and Fas-induced apoptosis, for example in amounts sufficient to inhibit Bax- and Fas-induced apoptosis in a subject, such as a human, who suffers from unwanted apoptosis. The compositions can be used in subjects who suffer from a condition such as a cancer, autoimmune or neurodegenerative disease, for example diabetes, multiple sclerosis or retinal degeneration, which is characterized by unwanted apoptosis. Alternatively, the disclosed compositions can be used for promoting apoptosis in a subject, such as a human, in whom apoptosis is desired. The compositions can be used in subjects who suffer from a condition such as cancer.

Also disclosed herein are methods for detecting an enhanced susceptibility of a subject to disease associated with abnormal apoptosis, by detecting a deletion of or within a VIAF gene, detecting other mutations of a VIAF gene and/or the abnormal expression such as a decrease or absence of VIAF protein in cells of a subject, such as a human. For example, in an extreme case, a total absence of VIAF protein may be detected. The disease may be cancer in which apoptosis is

abnormally decreased, or an autoimmune disease, or a neurodegenerative disease in which apoptosis is abnormally increased. In certain embodiments, a mutation (such as a substitution, insertion or deletion) of or in a VIAF gene can be detected by incubating a nucleic acid, such as an oligonucleotide, with the nucleic acid of the cell under conditions such that the oligonucleotide will specifically hybridize to a VIAF gene present in the nucleic acid to form an oligonucleotide:VIAF gene complex, and then detecting an increase or decrease of oligonucleotide:VIAF complexes, wherein the decrease of said complexes indicates a mutation (such as a deletion of or within) the VIAF gene. In an extreme case, this mutation may be a total absence of the VIAF gene. The present invention also provides methods for detecting the presence of VIAF protein in a cell by incubating a specific binding agent of the present invention with proteins of the cell under conditions such that the specific binding agent will specifically bind to a VIAF protein present in the cell to form a specific binding agent:VIAF protein complex, and detecting an increase or decrease (or quantity) of specific binding agent:VIAF protein complexes, including a total absence of the VIAF protein.

In another embodiment, VIAF biological activity can be supplied to a cell which has lost its VIAF biological activity, for example by a mutation of the VIAF gene, for example by a deletion of all or a portion of a VIAF gene, by introducing a VIAF gene into the cell so that the VIAF gene is expressed in the cell. In specific embodiments, the VIAF biological activity is supplied to treat a disease of abnormal apoptosis.

Also disclosed herein are methods for decreasing apoptosis in a cell by increasing the level of VIAF biological activity, which prevents the cell from undergoing apoptosis. In one embodiment, the cell is a neuron which has decreased VIAF protein expression or the cell has decreased VIAF biological activity or expression, relative to VIAF biological activity or expression in a same tissue type that is undergoing apoptosis. In certain embodiments, increasing the level of VIAF biological activity can be achieved by exposing the cell to a therapeutically effective amount of any of the VIAF proteins (including fragments and variants) disclosed herein. In other embodiments, the methods for decreasing apoptosis can be used to treat a disease of abnormal apoptosis, for example, autoimmune and neurodegenerative diseases, such as diabetes, multiple sclerosis and retinal degeneration.

Methods are also provided for inducing apoptosis in a subject by administering a therapeutically effective amount of an antisense oligonucleotide disclosed herein sufficient to induce apoptosis (including increased apoptosis) in the subject, for example a human. In a particular embodiment, inducing apoptosis treats a disease of insufficient apoptosis. In another embodiment, the antisense oligonucleotide specifically inhibits expression of VIAF protein, for example for diseases associted with excess apoptosis. The antisense molecule may include at least 20 contiguous nucleotides of a sequence that is complementary to at least a portion of an RNA

transcript of a VIAF gene, and is hybridizable to the RNA transcript, such as SEQ ID NOs 1, 3, 5 or 7.

In another embodiment, methods for treating a disease caused by a mutation in SEQ ID NOS 1, 3, 5, or 7, or a complementary strand, by supplying a therapeutically effective amount of a polypeptide product or the nucleic acid, are disclosed.

In yet another embodiment, a method is disclosed of inhibiting Bax- and Fas-induced apoptosis in a subject by administering a therapeutically effective amount of a purified protein having VIAF biological activity, or a nucleic acid which can express a protein having VIAF biological activity. In particular embodiments, the purified protein is any protein disclosed herein, which has VIAF biological activity.

The foregoing and other objects, features, and advantages disclosed herein will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the protein sequence of a human VIAF and alignment with sequences of some VIAF homologs in other species. Identical amino acids are shaded in gray.

FIG. 2 shows a predicted structure for a human VIAF protein having the amino acid sequence shown in FIG 1.

FIG. 3 is a digital image of a Northern blot showing VIAF mRNA expression in several tissues. As a control, the blot was probed for β-actin (lower panel).

FIG. 4 is a digital image of a dried SDS-polyacrylamide gel, showing the results of a precipitation experiment demonstrating that VIAF associates with Op-IAP *in vitro*.

FIG. 5 is a digital image of a western blot showing that endogenous VIAF and XIAP proteins interact *in vivo*.

FIG. 6 is a digital image of a western blot showing that the BIR domain of XIAP is necessary and sufficient for association with VIAF. The *in vitro* translated protein prior to precipitation is shown as input lanes.

FIG. 7 is a digital image of a western blot showing that VIAF interacts with XIAP, c-IAP1 and c-IAP2.

FIG. 8A is a graph showing that VIAF protects from Bax-induced apoptosis. Results shown are representative of three independent experiments.

FIG. 8B is a graph showing that VIAF protects from and Fas/Apo-1/CD95-induced apoptosis. Results shown are representative of three independent experiments.

FIG. 9 is a digital image of protein gels, and a bar graph quantitating the image, showing the synergistic effect of VIAF and XIAP on JNK activation.

FIG. 10 is a digital image of a Western blot showing that dexamethasone-induced VIAF expression in murine thymocytes is abrogated by ZVAD-fmk.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO 1 shows a nucleotide sequence of a human VIAF cDNA, GenBank Accession No. AF110511.

SEQ ID NO 2 shows an amino acid sequence of a human VIAF protein, GenBank Accession No. AF110511.

SEQ ID NO 3 shows a nucleotide sequence of a mouse VIAF cDNA, GenBank Accession No. AF110512.

SEQ ID NO 4 shows an amino acid sequence of a mouse VIAF protein, GenBank Accession No. AF110512.

SEQ ID NO 5 shows a nucleotide sequence of a Zebrafish VIAF cDNA, GenBank Accession No. AF175204.

SEQ ID NO 6 shows an amino acid sequence a Zebrafish VIAF protein, GenBank Accession No. AF175204.

SEQ ID NO 7 shows a nucleotide sequence of a *Drosophila* VIAF cDNA, GenBank Accession No. AF110513.

SEQ ID NO 8 shows an amino acid sequence a *Drosophila* VIAF protein, GenBank Accession No. AF110513.

SEQ ID NO 9 shows a nucleotide sequence of a *S. cerevisiae* VIAF cDNA, GenBank Accession No. AF110514.

SEQ ID NO 10 shows an amino acid sequence a *S. cerevisiae* VIAF protein, GenBank Accession No. AF110514.

SEQ ID NO 11 shows a nucleotide sequence of a sense primer that can be used to amplify the human VIAF cDNA.

SEQ ID NO 12 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the human VIAF cDNA.

SEQ ID NO 13 shows a nucleotide sequence of a sense primer that can be used to amplify the mouse VIAF cDNA.

SEQ ID NO 14 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the mouse VIAF cDNA.

SEQ ID NO 15 shows a nucleotide sequence of a sense primer that can be used to amplify the zebrafish VIAF cDNA.

SEQ ID NO 16 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the zebrafish VIAF cDNA.

SEQ ID NO 17 shows a nucleotide sequence of a sense primer that can be used to amplify the *Drosophila* VIAF cDNA.

SEQ ID NO 18 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the *Drosophila* VIAF cDNA.

SEQ ID NO 19 shows a nucleotide sequence of a sense primer that can be used to amplify the *S. cerevisiae* VIAF cDNA.

SEQ ID NO 20 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the *S. cerevisiae* VIAF cDNA.

SEQ ID NO 21 shows an amino acid sequence that can be used to generate a polyclonal antibody that recognizes the N-terminal region of VIAF.

SEQ ID NO 22 shows an amino acid sequence that can used to generate a polyclonal antibody that recognizes the C-terminal region of VIAF.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Abbreviations and Definitions

The following definitions and methods are provided to better define the materials and methods disclosed herein and to guide those of ordinary skill in the art in the practice of the materials and methods disclosed herein. As used herein (including the appended claims), the singular forms "a" or "an" or "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protein" includes a plurality of such proteins and reference to "the antibody" includes reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abnormal apoptosis: An amount of apoptosis, for example too much, too little, or both. For example, the amount of apoptosis associated with a disease of abnormal apoptosis can be compared with normal cells having normal apoptosis, for example cells of the same tissue type which do not have the disease. The amount of apoptosis can be determined using the methods described in EXAMPLE 9.

Animal protein: A higher eukaryotic protein, for example a protein expressed in mammals, fish, and *Drosophila*. The animal protein can be isolated from the animal, or expressed

recombinantly, for example in bacteria. Excludes lower eukaryotic proteins, for example, yeast, for example *S. cerevisiae*.

Antisense molecules or antisense oligonucleotides: Nucleic acid molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA (Weintraub, *Scientific American* 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double stranded. In one embodiment, the antisense oligomer is about 15 nucleotides, which are easily synthesized. The use of antisense molecules to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.* 172:289, 1988).

Therapeutically effective antisense molecules are characterized by their ability to inhibit the expression of VIAF. Complete inhibition is not necessary for therapeutic effectiveness, some oligonucleotides will be capable of inhibiting the expression of VIAF by at least 15%, 30%, 40%, 50%, 60%, or 70%.

Therapeutically effective antisense molecules are additionally characterized by being sufficiently complementary to VIAF encoding nucleic acid sequences. As described below, sufficient complementary means that the therapeutically effective oligonucleotide or oligonucleotide analog can specifically disrupt the expression of VIAF, and not significantly alter the expression of genes other than VIAF.

Calpain Inhibitor I: An inhibitor of calpain I, II, cathepsin B and cathepsin L. It inhibits: neutral cysteine proteases and the proteasome; apoptosis in thymocytes; and the proteolysis of IκBα and IκBα by the ubiquitin-proteasome complex.

Cancer: Malignant neoplasm that has undergone characteristic anaplasia with loss of differentiation, increased rate of growth, invasion of surrounding tissue, and is capable of metastasis.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

Chemical synthesis: The artificial means by which one can make a protein or peptide, for example as described in EXAMPLE 23.

Deletion: The removal of a sequence of DNA, the regions on either side being joined together.

DNA: Deoxyribonucleic acid. DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules

code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

Isolated: An "isolated" biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Lactacystin: A cell-permeable and irreversible proteasome inhibitor. It is a *Streptomyces* metabolite that acts as a highly specific inhibitor of the 20S proteasome, and blocks proteasome activity by targeting the catalytic β-subunit.

Malignant: Cells which have the properties of anaplasia invasion and metastasis.

Mimetic: A molecule (such as an organic chemical compound) that mimics the activity of a protein, such as the biological activity of VIAF. Peptidomimetic and organomimetic embodiments are within the scope of this term, wherein the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid sidechains in the peptide, resulting in such peptido- and organomimetics of the peptides having substantial specific inhibitory activity. For computer modeling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press: Buffalo Grove, IL, pp. 165-174 and Principles of Pharmacology (ed. Munson, 1995), chapter 102 for a description of techniques used in computer assisted drug design. Example 22 describes other methods which can be used to generate mimetics.

Mutant VIAF gene: A mutant form of a VIAF gene which in some embodiments is associated with disease, for example: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; B-cell acute lymphoblastic leukemia; diabetes mellitus, transient neonatal diabetes; and insulin dependent diabetes.

Mutant VIAF RNA: The RNA transcribed from a mutant VIAF gene.

Mutant VIAF protein: The protein encoded by a mutant VIAF gene.

Neoplasm: Abnormal growth of cells.

N rmal cells: Non-tumor, non-malignant cells.

Olig nucleotide: A linear polynucleotide sequence of up to about 200 nucleotide bases in length, for example a polynucleotide (such as DNA or RNA) which is at least 6 nucleotides, for example at least 15, 25, 50, 100 or even 200 nucleotides long.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into protein.

Ortholog: Two nucleotide sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

PCR (polymerase chain reaction): Describes a technique in which cycles of denaturation, annealing with primer, and then extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful herein are conventional. *Remington's Pharmaceutical Sciences*, by Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the DNA, RNA, proteins, and antibodies herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol, ethanol, combinations thereof, or the like, as a vehicle. The carrier and composition can be sterile, and the formulation suits the mode of administration. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, sodium saccharine, cellulose, magnesium carbonate, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

Probes and primers: Nucleic acid probes and primers may readily be prepared based on the amino acid sequences provided herein. A probe comprises an isolated nucleic acid attached to a

detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989) and Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences (1987).

Primers are short nucleic acids, such as DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), Ausubel *et al.*, 1987, and Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, 1990, Innis *et al.* (eds.), 21-27, Academic Press, Inc., San Diego, California. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Polynucleotide: A linear nucleic acid sequence of any length. Therefore, a polynucleotide includes molecules which are 15, 50, 100, 200 (oligonucleotides) and also nucleotides as long as a full length cDNA.

Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. For example, a preparation is purified when the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

Sample: Includes biological samples containing genomic DNA, RNA, or protein obtained from the cells of a subject, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, amniocentesis samples and autopsy material.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or orthologs of nucleic acid or amino acid sequences will possess a relatively high degree of sequence identity when aligned using standard methods. This homology will be more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (e.g., human and chimpanzee sequences), compared to species more distantly related (e.g., human and *C. elegans* sequences). Typically, VIAF orthologs are at least 50% identical at the nucleotide level and at least 50% identical at the amino acid level when comparing orthologus sequences.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet et al., *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al*,. *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

Homologs of the VIAF proteins are typically characterized by possession of at least 60% sequence identity counted over full-length alignment with the amino acid sequence of human VIAF using the NCBI Blast 2.0, gapped blastp set to default parameters. Queries searched with the blastn program are filtered with DUST (Hancock, and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. Alternatively, one may manually align the sequences and count the number of identical amino acids. This number divided by the total number of amino acids in the reference sequence multiplied by 100 results in the percent identity.

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer

than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 70%, 75%, 80%, 90%, 95%, 98%, 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85%, 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site.

One of ordinary skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. Provided herein are the peptide homologs described above, as well as nucleic acid molecules that encode such homologs.

An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5 C to 20 C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence remains hybridized to a perfectly matched probe or complementary strand. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* ((1989) In *Molecular Cloning: A Laboratory Manual*, CSHL, New York) and Tijssen ((1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* Part I, Chapter 2, Elsevier, New York). Nucleic acid molecules that hybridize under stringent conditions to a VIAF gene sequence will typically hybridize to a probe based on either an entire VIAF gene or selected portions of the gene under wash conditions of 2x SSC at 50 C. A more detailed discussion of hybridization conditions is presented in EXAMPLE 13.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein.

Such homologous peptides may, for example, possess at least 75%, 80%, 90%, 95%, 98%, or 99% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs may, for example, possess at least 75%, 85% 90%, 95%, 98% or 99% sequence identity over short windows of 10-20 amino acids. Methods for determining sequence identity over such short windows can be found at the NCBI web site. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it

is entirely possible that strongly significant homologs or other variants could be obtained that fall outside of the ranges provided.

The disclosure provides not only the peptide homologs that are described above, but also nucleic acid molecules that encode such homologs.

An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

Specific binding agent: An agent that binds substantially only to a defined target. As used herein, the term "VIAF peptide specific binding agent" includes anti-VIAF peptide antibodies and other agents that bind substantially only to the VIAF peptide. The antibodies may be monoclonal or polyclonal antibodies that are specific for the VIAF peptide, as well as immunologically effective portions ("fragments") thereof. In one embodiment, the antibodies used herein are monoclonal antibodies (or immunologically effective portions thereof) and may also be humanized monoclonal antibodies (or immunologically effective portions thereof). Immunologically effective portions of monoclonal antibodies include Fab, Fab', $F(ab')_2$, Fabc and Fv portions (for a review, see Better and Horowitz, *Methods. Enzymol.* 178:476-96, 1989). Anti-inhibitory peptide antibodies may also be produced using standard procedures described in a number of texts, including *Antibodies, A Laboratory Manual* by Harlow and Lane, Cold Spring Harbor Laboratory (1988).

The determination that a particular agent binds substantially only to the VIAF peptide may readily be made by using or adapting routine procedures. One suitable *in vitro* assay makes use of the Western blotting procedure (described in many standard texts, including *Antibodies, A Laboratory Manual* by Harlow and Lane). Western blotting may be used to determine that a given VIAF peptide binding agent, such as an anti-VIAF peptide monoclonal antibody, binds substantially only to the VIAF protein.

Specifically hybridizable and specifically complementary: Terms which indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions in which specific binding is

desired, for example under physiological conditions in the case of *in vivo* assays. Such binding is referred to as "specific hybridization." See EXAMPLE 13 for hybridization conditions.

Subject: Living multicellular vertebrate organism, a category which includes, both human and veterinary subjects, for example, mammals, fish, and birds.

Sufficient complementarity: When used, indicates that a sufficient number of base pairs exist between the oligonucleotide and the target sequence to achieve detectable binding, and disrupt expression of gene products (such as VIAF). When expressed or measured by percentage of base pairs formed, the percentage complementarity that fulfills this goal can range from as little as about 50% complementarity to full, (100%) complementary. In general, sufficient complementarity is at least about 50%. In one embodiment, sufficient complementarity is at least about 75% complementarity. In another embodiment, sufficient complementarity is about 90% or about 95% complementarity. In yet another embodiment, sufficient complementarity is about 98% or 100% complementarity.

A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz *et al. Methods Enzymol* 100:266-285, 1983, and by Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Target sequence: A portion of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) or RNA that upon hybridization to an therapeutically effective oligonucleotide or oligonucleotide analog results in the inhibition of VIAF expression. Either an antisense or a sense molecule can be used to target a portion of dsDNA, since both will interfere with the expression of that portion of the dsDNA. The antisense molecule can bind to the plus strand, and the sense molecule can bind to the minus strand. Thus, target sequences can be ssDNA, dsDNA, and RNA.

An oligonucleotide "binds" or "stably binds" to a target nucleic acid if a sufficient amount of the oligonucleotide forms base pairs or is hybridized to its target nucleic acid, to permit detection of that binding. Binding can be detected by either physical or functional properties of the target:oligonucleotide complex. Binding between a target and an oligonucleotide can be detected by any procedure known to one skilled in the art, including both functional and physical binding assays. Binding may be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation and the like.

Physical methods of detecting the binding of complementary strands of DNA or RNA are well known in the art, and include such methods as DNase I or chemical footprinting, gel shift and affinity cleavage assays, Northern blotting, dot blotting and light absorption detection procedures. For example, one method that is widely used, because it is so simple and reliable, involves observing a change in light absorption of a solution containing an oligonucleotide (or an analog)

and a target nucleic acid at 220 to 300 nm as the temperature is slowly increased. If the oligonucleotide or analog has bound to its target, there is a sudden increase in absorption at a characteristic temperature as the oligonucleotide (or analog) and target disassociate or melt.

The binding between an oligomer and its target nucleic acid is frequently characterized by the temperature (T_m) at which 50% of the oligomer is melted from its target. A higher (T_m) means a stronger or more stable complex relative to a complex with a lower (T_m).

Therapeutically Effective Amount: A concentration of VIAF, for example an amount that is effecitve to modulate (increase or decrease) the anti-apoptotic and signaling properties of the IAP family in a subject to whom it is administered. In particular detailed examples, it is an amount required to inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example more than about 1.5 fold, for example more than about 2-fold. In other examples, it is an amount required when co-expressed with suboptimal quantities of XIAP, will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli. In yet other examples, it is an amount required to synergistically stimulate c-Jun N-terminal kinase activity. Such inhibition will decrease (including preventing) apoptosis in a cell, such as the cell of a patient.

The therapeutically effective amount also includes a quantity of VIAF protein sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to improve signs and/or symptoms a disease of abnormal apoptosis, for example by modulating the anti-apoptotic and signaling properties of the IAP family.

An effective amount of VIAF may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount of VIAF will be dependent on the source of VIAF applied (i.e. VIAF isolated from a cellular extract versus a chemically synthesized and purified VIAF, or a variant or fragment that may not retain full VIAF activity), the subject being treated, the severity and type of the condition being treated, and the manner of administration of VIAF. For example, a therapeutically effective amount of VIAF can vary from about 0.01 mg/kg body weight to about 1 g/kg body weight.

The VIAF protein disclosed herein has equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals (e.g. humans, apes, dogs, cats, horses, and cows) that require modulation of the anti-apoptotic and signaling properties of the IAP family activity that is susceptible to VIAF-mediated modulation.

Therapeutically effective dose: A dose sufficient to modulate the apoptotic and signaling properties of the IAP family, resulting in a regression of a pathological condition, or which is capable of relieving signs or symptoms caused by the condition, such as cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; B-cell acute lymphoblastic leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes.

Transf rmed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Transgenic Cell: Transformed cells which contain foreign, non-native DNA.

Tumor: A neoplasm

Variants or fragments or fusion proteins: The production of VIAF proteins can be accomplished in a variety of ways (for example see EXAMPLES 6, 13, 14 amd 29). DNA sequences which encode for the protein or fusion protein, or a fragment or variant of the protein, can be engineered such that they allow the protein to be expressed in eukaryotic cells, bacteria, insects, and/or plants. In order to accomplish this expression, the DNA sequence can be altered and operably linked to other regulatory sequences. The final product, which contains the regulatory sequences and the therapeutic protein, is referred to as a vector. This vector can then be introduced into the eukaryotic cells, bacteria, insect, and/or plant. Once inside the cell the vector allows the protein to be produced.

One of ordinary skill in the art will appreciate that the DNA can be altered in numerous ways without affecting the biological activity of the encoded protein. For example, PCR may be used to produce variations in the DNA sequence which encodes VIAF. Such variants may be variants that are optimized for codon preference in a host cell that is to be used to express the protein, or other sequence changes that facilitate expression.

Two types of cDNA sequence variant may be produced. In the first type, the variation in the cDNA sequence is not manifested as a change in the amino acid sequence of the encoded polypeptide. These silent variations are simply a reflection of the degeneracy of the genetic code. In the second type, the cDNA sequence variation does result in a change in the amino acid sequence of the encoded protein. In such cases, the variant cDNA sequence produces a variant polypeptide sequence. In order to optimize preservation of the functional and immunologic identity of the encoded polypeptide, any such amino acid substitutions may be conservative. Conservative substitutions replace one amino acid with another amino acid that is similar in size, hydrophobicity, etc.

Variations in the cDNA sequence that result in amino acid changes, whether conservative or not, are minimized to enhance preservation of the functional and immunologic identity of the encoded protein. The immunologic identity of the protein may be assessed by determining whether it is recognized by an antibody to VIAF; a variant that is recognized by such an antibody is immunologically conserved. In particular embodiments, any cDNA sequence variant will introduce no more than 20, for example fewer than 10 amino acid substitutions into the encoded polypeptide. Variant amino acid sequences can, for example, be 80%, 90% or even 95% identical to the native

amino acid sequence. In other embodiments, the VIAF protein has less than 275 amino acids, for example less than 250, 240, or 237 amino acids. Alternatively, the VIAF protein may have 237, 236, 200, 150, or 100 amino acids.

Conserved residues in the same or similar proteins from different species can also provide guidance about possible locations for making substitutions in the sequence. A residue which is highly conserved across several species is more likely to be important to the function of the protein than a residue that is less conserved across several species.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

VIAF Biological Activity: Such biological activity includes the ability to modulate (and especially increase) the anti-apoptotic and signaling properties of the IAP family. Alternatively (or in addition), the protein has the ability to inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example by a factor of at least 1.5, for example at least 2, (where the factor refers to at least approximately double the cell viability using the assay described in EXAMPLE 9). Alternatively (or in addition), the protein has the ability to bind, interact with OpIAP, XIAP, c-IAP1 and c-IAP2 as determined by the assays described in EXAMPLES 7 and 8. In other embodiments, co-expression of the protein with suboptimal quantities of XIAP will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli (where the factor refers to the increase in cell viability using the assay described in EXAMPLE 9). In yet another embodiment, the protein can synergistically stimulate c-Jun N-terminal kinase activity using the methods described in EXAMPLE 10. In very particular embodiments, the biological activity includes any combination of the characteristics in this paragraph, or all of them.

VIAF cDNA: The *VIAF* cDNA is functionally defined as a cDNA molecule which encodes a protein having VIAF biological activity. The *VIAF* cDNA is derived by reverse transcription from the mRNA encoded by the *VIAF* gene and lacks internal non-coding segments and transcription regulatory sequences found in the *VIAF* gene.

VIAF fusion protein: A fusion protein comprising a VIAF protein (or variants, mutants, or fragments thereof) linked to other amino acid sequences.

VIAF gene: A gene which encodes a protein having VIAF biological activity. The definition of an VIAF gene includes the various sequence polymorphisms that may exist in other species.

VIAF protein: The protein encoded by VIAF cDNA. This protein may be functionally characterized by its biological ability as described above. VIAF proteins include the full-length cDNA transcript (SEQ ID NOs 2, 4, 6, 8 and 10), as well as shorter peptides which retain VIAF biological activity.

ZVAD-fmk: Tripeptide derivativebenzyloxycarbonyl-Val-Ala-Asp (Ome)-fluoromethyl ketone. A highly specific, cell-permeable and irreversible inhibitor of caspase-1- like proteases. Inhibits Fas-mediated apoptosis in Jurkat cells.

Additional definitions of common terms in molecular biology may be found in Lewin, B. *Genes V* published by Oxford University Press.

EXAMPLE 1

Cloning of a Human VIAF cDNA

A human VIAF was cloned using the yeast two-hybrid system. Yeast two-hybrid library screening and analysis were performed as described in the Matchmaker protocol provided by Clontech (Palo Alto, CA) using the yeast strain Y153. OpIAP (baculoviral IAP) was used as a bait to perform a yeast two-hybrid screen of a B cell lymphoma library. The known interaction between CD30 and TRAF2 was used as a positive control.

Competent Y153 yeast cells were transformed with the appropriate pAS1 and pACT plasmid DNA derivatives (for library screening 30 μg of each plasmid) containing fish sperm carrier DNA. To transform the yeast cells, the DNA and yeast were combined with polyethylene glycol/LiOAc and incubated one hour at 30°C, then for 25 minutes at 42°C. The yeast cells were then plated on plates lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole (3-AT) and plates lacking leucine and tryptophan. After three days at 30°C, colonies from plates lacking leucine, tryptophan, and histidine and containing 3-AT were transferred to filters and assayed for β-galactosidase activity. In all cases, colonies from the plates lacking leucine and tryptophan gave identical results. In general, β-galctosidase activity was apparent within one hour, but the filters were allowed to incubate for 24 hours. In all assays, transformants were tested for both the ability to grow on medium lacing histidine and containing 3-AT and for β-galactosidase expression.

Three overlapping, independent groups of clones, whose in-frame coding sequences possessed identical C-termini, were isolated. These clones interacted specifically with OpIAP but not a variety of negative controls (Table 1), including the GAL4 DNA binding domain alone (Durfee *et al.*, 1993, *Genes Dev.* 7:555-69), the cytoplasmic domain of the TNF receptor family member CD30 (Gedrich *et al.*, 1996, *J. Biol. Chem.* 271:12852-8), and the yeast SNF1-associated protein SNF4 (Durfee *et al.*, 1993, *Genes Dev.* 7:555-69). The OpIAP-interacting clones also scored negative against the homologous AcIAP, a non-functional IAP from *Autographa californica* nuclear polyhedrosis virus (Table 1), indicating that the ability of OpIAP to interact with this protein might be an important determinant of anti-apoptotic function.

Table 1: VIAF Interacts with OpIAP but not AcIAP

		Prey		
		Vector	VIAF	TRAF2
Bait	Vector	-	-	-
	CD30	-	-	+++
	OpIAP	-	+++	-
	AcIAP	-	-	-

The sequence obtained from the yeast two-hybrid screen was used to perform an EST database search. The full-length EST cDNA was subcloned into pBlueScript SK(+) (Stratagene), using EcoRI and NotI as cloning sites. All cDNA clones were fully sequenced in both directions. The resulting cDNA sequence (SEQ ID NO 1) encodes a novel 239 amino acid protein (SEQ ID NO 2) with a predicted molecular weight of 28 kDa (FIG. 1) which was designated VIAF (viral IAP-associated factor). The smallest VIAF clone obtained through two-hybrid screening contained the C-terminal 128 residues (amino acids 112 to 239 of SEQ ID NO 2) demonstrating this region is necessary and sufficient for interaction with OpIAP.

The VIAF open reading frame exhibited no obvious homology to caspases, IAPs or other proteins currently known to be involved in the regulation or execution of the apoptotic pathway. VIAF was has limited homology (27% over 165 residues) to phosducin, a cytosolic protein that interacts with the βγ subunits of G proteins. Immunofluorescence studies using 293 cells transfected with epitope-tagged VIAF demonstrated that VIAF is exclusively cytoplasmic.

Human VIAF maps to chromosome location 6q23.1 to 6q24.3. Several diseases associated with this region include: heterocellular hereditary persistence of fetal hemoglobin (142470); deafness (601316); cardiomyopathy (602067); B-cell acute lymphoblastic leukemia (designated as the six-twelve leukemia gene; 602532); diabetes mellitus, transient neonatal diabetes (601410) and insulin dependent diabetes (606320). Numbers in parenthesis correspond to the Online Mendelian Inheritance in Man Gene Map numbers. Additional information can be found on the Internet through the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894), for example the website of the Online Mendelian Inheritance in Man Gene Map. These are example of diseases associated with abnormal apoptosis. Leukemia, for example, is a malignancy in which malignant cells do not undergo normal apoptosis. Cardiomyopathy and diabetes are examples of conditions in which cells (such as myocardial cells or pancreatic islet cells) undergo unwanted apoptosis.

EXAMPLE 2

Cloning of VIAF in Other Organisms

A VIAF gene was cloned in several different organisms including: mouse, zebrafish, *Drosophila* and yeast (*S. cerevisiae*). Specific primers were designed to perform PCR to obtain the VIAF homologues.

The following primers were used to clone VIAF in several species: human VIAF sense primer: ATAGGATCCATGGAGGACCCCAACGCAGACACTG (SEQ ID NO 11); human antisense primer: 5'-AATATCGATCCAGACAATTTGTCACAAGAAAGTTCG-3' (SEQ ID NO 12); mouse VIAF sense primer: 5'-AATGGATCCATGCAGGACCCCAATGCAGACACC-3' (SEQ ID NO 13); mouse antisense primer: 5'- ATTATCGATTCAAAGGTTCCATCACTGCCA-3' (SEQ ID NO 14); zebrafish sense primer: 5'-ATAGGATCCATGCAGGACCCAAAC GACACCGAGTGGAAC-3' (SEQ ID NO 15); zebrafish antisense-primer: 5'-AATATCGATCGTGGGCAGGTTGCGGTCGGGGTAGTTGGG -3' (SEQ ID NO 16); *Drosophila* VIAF: sense primer: 5'-TAAATCGATATGCAGGACCCAAACGAAGATACC-3' (SEQ ID NO 17); *Drosophila* antisense primer: 5'-ATAATCGATTGCCGGTTTTGGATTGGG-3' (SEQ ID NO 18); yeast VIAF sense primer: 5'-AATGGATCCATGGAGAATGAACCAATGTTTCAGG-3' (SEQ ID NO 19); and yeast antisense primer: 5'-ATAGCGGCCGCCTGTAAATAAGGAATATTGGCA-3' (SEQ ID NO 20).

For all of the VIAF cloning experiments, the following PCR conditions were used: 94°C for 5 minutes; 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 2 minutes (30 cycles); 72°C for 10 minutes. The mouse and yeast homologues were amplified using DNA from a mouse T cell library and yeast genomic DNA, respectively. The *Drosophila* library used was the Matchmaker 2hybrid library, (Clontech, Cat # IL 4002AH), and the missing 3'-end was amplified with Marathon cDNA Amplification Kit, (Clontech Cat # K1802-1). The zebrafish cDNA was cloned using EST clone #fb37bO3 (Research Genetics, AL).

VIAF cDNA was cloned in several species, and the protein encoded by the cDNA determined: mouse (SEQ ID NOS 3 and 4), zebrafish (SEQ ID NOS 5 and 6), *Drosophila* (SEQ ID NOS 7 and 8), and yeast (SEQ ID NOS 9 and 10) were cloned. The approximate percent identity to the human VIAF nucleic acid sequence (SEQ ID NO 1) to the mouse, zebrafish, *Drosophila* and yeast VIAF nucleic acid sequences is 84.9%, 70.8%, 59.0%, and 48%, respectively.

As shown in FIG. 1, there is a high degree of conservation of the VIAF amino acid sequence between different species. The average percent identity to the human clone between the mouse, zebrafish, *Drosophila* and yeast homologues at the amino acid level is 90%, 74.8%, 51.8% and 32.3% similar, respectively.

EXAMPLE 3

Structure of VIAF

The structure of VIAF predicted from the amino acid sequences of the proteins obtained in EXAMPLES 1 and 2 is shown in FIG. 2. There is a predicted coiled coil domain (amino acids 23-89), predicted SHC SH2 domain binding site (amino acids 48-52), two predicted ATP binding sites (amino acids 83-96 and 100-111) and a predicted OpIAP interaction domain (amino acids 111-239). The amino acid numbers refer to those shown in SEQ ID NO 2.

Additional studies were conducted to determine if VIAF is a phosphoprotein. Human embryonic kidney cells (293 cells) were transfected with 2 μg of full-length human VIAF (SEQ ID NO 1) cloned into the expression vector pEBB (Mayer *et al.*, 1995, *Curr.Biol.* 5:296-305), or the vector alone. Six hours after transfection, cells were incubated for 12 hours in phosphate-free RPMI 1640 and then labeled for 24 hours with [^{32}P]-orthophosphate. Radioactive media was removed, cells were washed five times with serum-free RPMI 1640 and then lysed with Triton-X-100 containing buffer. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and labeled proteins were visualized by autoradiography. The results demonstrated that VIAF is a phosphoprotein.

EXAMPLE 4

Northern Analysis of VIAF RNA Expression

VIAF mRNA expression was analyzed using Northern blot analyses. Full-length human VIAF cDNA (SEQ ID NO 1) was labeled with [α^{32}P]dATP using the Rediprime II kit (Amersham Pharmacia Biotech, Piscataway, NJ, Cat # RPN 1633) as instructed by the manufacturer. Briefly, the cDNA (100 ng) was combined with α-[^{32}P] dATP (4 μl) and the mixture incubated for 30 minutes at 37°C. Unincorporated radiolabel was separated from the labeled cDNA by centrifuging it through a G50-Sephadex column. The radiolabeled human VIAF cDNA was used to probe a multiple adult human tissue blot (Clontech). Hybridization was carried out under high stringency conditions according to the manufacturers instructions. The blot was prehybridized for 30 minutes at 68°C with ExpressHyb solution (Clontech), and then hybridized for one hour at 68°C in ExpressHyb. The blot was washed four times at room temperature (RT) with 2x SSC, 0.1% SDS and two times at 50°C with 0.1x SSC, 0.1% SDS. The blot was exposed overnight at -80°C with intensifier screens. As a control, the blot was incubated with a radioactive probe for β-actin. The cDNA for β-actin (Clontech) was radiolabeled as described above using the Rediprime II kit.

As shown in FIG. 3, Northern blot analyses revealed a major band of approximately 1.2 kb with the VIAF probe, and a major band of 2.0 kb with the β-actin control probe. VIAF mRNA is ubiquitously expressed but its mRNA expression is most abundant in the testis, followed by vary and prostate. Expression of VIAF is minimal in the thymus.

EXAMPLE 5

Generation of Polyclonal Antibodies Against VIAF

The example describes a method used to generate polyclonal antibodies against human VIAF. Similar experiments can be used to produce polyclonal antibodies to VIAF from other species, such as zebrafish VIAF, using the protein sequences disclosed herein. In addition, similar experiments can be used to generate polyclonal antibodies that recognize other VIAF fragments, variants, or mutant forms thereof.

Rabbit polyclonal sera was raised against keyhole limpet hemocyanin (KLH)-conjugated peptides encoding the N- and C-termini of VIAF. The peptide sequence for the antibody against the N-terminal end was: (KLH)-MQDPNADTEWNDILR (SEQ ID NO 21) and the sequence for the C-terminal end was: (KLH)-RRSVLMKRDSDSEGD (SEQ ID NO 22).

The antibody titer was determined by ELISA assay with free peptide bound in solid phase (1 μg/well). Results were expressed as the reciprocal of the serum dilution that results in an OD_{405} of 0.2. Detection was obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

To demonstrate that the antibodies specifically recognized the VIAF protein, the antibodies were used to probe a membrane containing recombinant VIAF. Recombinant VIAF proteins (full-length cDNAs, SEQ ID NOs 1, 3, 5, and 9) were each cloned into a pEBB expression vector (Mayer *et al.*, 1995, *Curr.Biol.* 5:296-305). Expression of the VIAF cDNA using this vector results in a VIAF fusion protein containing a Flag-tag. The peBB-VIAF encoding vectors were transfected into 293 cells. After allowing expression of the recombinant VIAF, the cells were lysed (1% Triton-X-100, 1 mM EDTA, 100 mM NaCl, 50mM Tris-HCl [pH 8.0]) and the proteins resolved using SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, which was probed with a mixture of the polyclonal antibodies generated above. The membrane was subsequently incubated with the secondary antibody donkey anti-rabbit Ig, horseradish peroxidase-conjugated linked whole antibody (Amersham Pharmacia Biotech Cat # NA934). The VIAF proteins were visualized using Enhanced Chemiluminescence.

The VIAF polyclonal antibodies recognized recombinant Flag-tagged VIAF proteins (approximately 30 kDa) of all species tested (human, mouse, *Drosophila*) except yeast. As a negative control, samples containing only the pEBB-Flag vector alone were used. Since the polyclonal antibodies did not recognize the Flag-tag, this demonstrates that the antibodies specifically recognize the VIAF protein. In addition, this demonstrates that the cloned cDNAs encode native VIAF proteins.

VIAF-specific antibodies can be generated using the above method, or by using methods described in EXAMPLE 15.

EXAMPLE 6

Expression of Recombinant GST-VIAF

This example describes the expression of recombinant GST-VIAF in *E. coli*. Full-length human VIAF cDNA (SEQ ID NO 1) was cloned into a pGEX-2TK vector (Amersham Pharmacia Biotech) using the SmaI and EcoRI cloning sites. Expression of the VIAF cDNA using this vector results in a VIAF fusion protein containing a GST (glutathione-S-transferase) tag.

The plasmid was transformed into XL 1-Blue *E. coli* (Stratagene, La Jolla, CA). Bacteria were grown in 500 ml LB media until an OD_{600} of 0.6 was achieved. Cells were subsequently induced with 1 mM IPTG for three hours. The bacteria cells were then lysed and sonicated in 50 ml GST-lysis buffer (1% Triton-X-100, 1 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl [pH 8.0]). The resulting lysates were centrifuged at 6000 rpm for 30 minutes and supernatant incubated for two hours with 500 μl of a 50% slurry of Protein G beads (Amersham Pharmacia Biotech). The beads were washed five times with lysis buffer. These beads were used directly for the precipitation experiments described below.

Aliquots containing 10 μl beads were loaded onto a SDS-polyacrylamide gel and stained with Colloidal Blue Staining Kit (NOVEX, San Diego, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The VIAF proteins were detected using a mixture of the polyclonal antibodies generated in EXAMPLE 5. The blot was subsequently incubated with the secondary antibody donkey anti-rabbit Ig, horseradish peroxidase-conjugated linked whole antibody (Amersham Pharmacia Biotech, Cat # NA934). The gels were fluorographed with Enlightning solution (NEN, Boston, MA) and VIAF proteins visualized by autoradiography.

EXAMPLE 7

VIAF Interacts with OpIAP

This example describes a precipitation assay used to demonstrate that VIAF interacts with OpIAP *in vitro*. OpIAP (0.5 μg plasmid DNA) was *in vitro* translated from the Bluescript plasmid templates with ^{35}S-labeled methionine in rabbit reticulolysates (30 μCi/sample) using the TNT T7/T3-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions.

For GST precipitation experiments, VIAF was subcloned into pGEX-2TK (Amersham Pharmacia Biotech). GST-VIAF fusion protein and GST control protein were expressed in XL-1 *E. coli* (Stratagene, La Jolla, CA) and purified as described previously (Gedrich *et al.*, 1996, *J. Biol. Chem.* 271:12852-8, herein incorporated by reference). Coprecipitation experiments with *in vitro* translated proteins were performed as described previously (*Id.*), except that Triton buffer was used for washes (25 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10%

glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride and cocktail of protease inhibitors). The precipitated proteins were separated by SDS-PAGE. Gels were fluorographed with Enlightning solution (NEN, Boston, MA) and visualized by autoradiography.

As shown in FIG. 4, recombinant GST-VIAF, but not GST alone, associates with OpIAP *in vitro*. The input lanes are the total cell lysates, prior to precipitation, showing that the OpIAP proteins were *in vitro* translated.

EXAMPLE 8

Identification of IAPs which Interact with VIAF

As shown in EXAMPLE 7, VIAF interacts with baculoviral OpIAP *in vitro*. To determine if VIAF interacts with human IAP proteins, co-immunoprecipitation experiments were conducted.

The mammalian IAP XIAP (ILP-1) functions as a downstream inhibitor of cell death through its association and enzymatic inhibition of Caspase-3. To determine whether endogenous human VIAF and XIAP proteins interact, the following experiment was performed. Four semi-confluent 10 cm dishes (approximately 1.5 x 10^7 cells per plate) of 293 cells were washed twice with phosphate-buffered saline and then lysed for 10 minutes on ice in 500 μl per plate of Triton buffer supplemented with 1 mM sodium orthovanadate and 1 mM sodium fluoride. Lysates were pooled and incubated for two hours at 4°C together with a cocktail of VIAF polyclonal sera (see EXAMPLE 5) and precipitated for an additional hour with 50 μl of protein G agarose (Life Technologies, Rockville, MD). The precipitates were washed three times and immunoblotted with the anti-VIAF polyclonal antibodies or with an anti-XIAP monoclonal antibody (Transduction Laboratories, Lexington, KY). Blots were resolved using either horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies contained within the Enhanced Chemiluminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech). XIAP coimmunoprecipitated with VIAF polyclonal antibodies but not with preimmune sera, indicating that VIAF and XIAP normally co-associate in cells (FIG. 5).

To further examine the VIAF-XIAP interaction, GST-VIAF protein was tested in coprecipitation experiments using several ^{35}S-labeled deletion mutants of XIAP generated by *in vitro* translation and precipitated with GST-VIAF. A truncation mutant of XIAP encoding only the three N-terminal BIR (baculovirus IAP repeat) domains was efficiently precipitated with GST-VIAF, demonstrating that the BIRs are necessary and sufficient for interaction with VIAF (FIG. 6, 3 BIR). However, a deletion mutant lacking the three BIRs could also coprecipitate, although with much less efficiency (FIG. 6, Δ BIR), suggesting that more that one VIAF binding site may exist in XIAP. GST-VIAF did not coprecipitate Caspase-9, confirming the specificity of the VIAF-XIAP interaction.

To determine if human VIAF interacts with other mammalian IAP proteins, co-precipitation experiments were performed with GST-VIAF and *in vitro* translated ^{35}S-labeled XIAP, c-IAP1 and c-IAP2 proteins. These IAP proteins were efficiently precipitated by GST-VIAF (FIG. 7), but not by GST alone, indicating that VIAF can also associate with c-IAP1 and c-IAP2.

EXAMPLE 9

VIAF Regulates Bax- and Fas-Mediated Apoptosis

To determine whether VIAF could regulate apoptosis, an expression vector encoding VIAF was tested for its ability to regulate apoptosis induced by Bax and Fas.

For Bax-induced apoptosis assays, 293 cells were transfected by calcium phosphate precipitation for 15 hours with 0.5 μg of a plasmid encoding β-galactosidase, 0 - 4 μg of a plasmid encoding human VIAF (see EXAMPLE 3) together with: 2 μg pcDNA3 ( ), 1 μg Bax (■), 1 μg Bax and 0.1 μg XIAP (▼), or 1 μg Bax and 0.5 μg XIAP (●) (FIG. 8A). For Fas-induced apoptosis assays, 293 cells were transfected by calcium phosphate precipitation for 20 hours with 0.5 μg of a plasmid encoding β-galactosidase, 0 - 4 μg of a plasmid encoding human VIAF (see EXAMPLE 3) together with: 2 μg of pcDNA3 ( ), 2 μg of Fas (■), 2 μg of Fas and 0.1 μg XIAP (▼) or 2 μg of Fas and 0.5 μg of XIAP (●). The total amount of DNA was equalized with control vector. Cells death of transfected cells was counted by morphology. Viable cells are spread out and adherent to the cell culture dish. In contrast, apoptotic cells detach from the culture dish and have a rounded shape. In addition, the cell membrane of apoptotic cells is blebed and in later stages of apoptosis the cell dissolves into apoptotic bodies.

Transfection of VIAF into 293 cells inhibited Bax-mediated apoptosis in a dose-responsive manner (FIG. 8A), although this protection reached a plateau at two micrograms of transfected plasmid, indicating that VIAF exerts its effects through a cellular factor whose concentration is limiting. Coexpression of XIAP at levels which alone only partially protect against Bax were almost completely protective when coexpressed with VIAF (FIG. 8A). Similarly, VIAF enhanced the ability of XIAP to protect from Fas-mediated apoptosis (FIG. 8B). Almost identical results were obtained using VIAF and OpIAP. These results demonstrate that VIAF can substantially protect cells from both Fas- and Bax-induced apoptosis, and co-expression of VIAF with suboptimal quantities of XIAP confers almost complete protection from these stimuli.

EXAMPLE 10

VIAF and XIAP have a Synergistic Effect on JNK activation

In addition to their caspase-inhibitory properties, IAPs are also involved in the regulation of c-Jun N-terminal kinase (JNK). JNK is required for the protective role of XIAP (Sanna *et al.* 1998, *Proc. Natl. Acad. Sci. USA* 95:6015-20). To further investigate the mechanism of VIAF, the role of VIAF in these functions was examined.

Kinase assays were performed as follows. Cell lysate (100 μl) was incubated for one hour at 4°C on rotator with one μl of HA antibody (12CA5 Boehringer Mannheim) and 10 μl of protein A beads (Pierce) which were previously washed and resuspended in 100 μl of M2 buffer (20 mM Tris, pH 7.6; 0.5% NP40; 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.2 mM DTT, 0.05 mM PMSF, 20 mM beta-glycerophosphate, 0.1 mM sodium vandate). The beads were washed twice with M2 buffer followed by two washings with kinase buffer (20 mM Hepes, pH 7.5; 20 mM beta-glycerophosphate, 10 mM PNPP, 10 mM $MgCl_2$, 1 mM DTT, 50 μM sodium vanadate). The beads were incubated for 30 minutes in 50 μl kinase buffer containing 20 μM ATP and 0.5 μl gamma-ATP (0.5 μCi) and 2 μg GST-cjun. Samples were subjected to SDS-page and the gel dried and exposed to autoradiography. Activation of JNK was detected by phosphorylation of GST-c-jun (1-79) (FIG. 9, c-jun-P). Expression of VIAF and XIAP was confirmed by immunoblotting of lysates with antibodies to VIAF and XIAP as described in EXAMPLE 8. Quantitation of JNK activity was performed by phosphorimage analysis using Storm 840 and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

As shown in FIG. 9, coexpression of VIAF and XIAP synergistically induced JNK activation, indicating that that VIAF may exert its effects by potentiating the JNK-inducing properties of XIAP.

While both Fas and Bax induce the activation of Caspase-3, in 293 cells this is achieved through two distinct pathways (Hakem *et al.*, 1998, *Cell* 94:339-52; Kuida *et al.*, 1998, *Cell* 94:325-37; Varfolomeev *et al.*, 1998, *Immunity* 9:267-76). Fas-induced death occurs in a manner which does not require activation of Caspase-9 and which is not blocked by dominant negative Caspase-9, while Bax-induced death occurs through the activation of a Caspase-9:Apaf1 holoenzyme complex, and therefore is blocked by expression of dominant negative Caspase-9. Taken together, these data indicate that the point of action of VIAF is downstream in the apoptotic cascade after the Fas- and Bax-induced pathways have converged, and that VIAF may function to enhance the pro-survival properties of XIAP or of other endogenous IAP proteins.

EXAMPLE 11

Characterization of Endogenous VIAF in Murine Thymocytes

This example describes experiments conducted to determine the role of endogenous cellular VIAF in apoptosis. The cells used in this example were freshly isolated, not from a cell line. Similar experiments can be used to examine VIAF expression in other samples, such as biopsies or others.

Isolation of Murine Thymocytes

Mice (B6 black mice no older than three months in age) were sacrificed and sterilized with ethanol. The thymus was removed and placed in RPMI media containing 10% FCS. After

transferring the thymus into a new petri dish to eliminate the blood cells, the thymus was cut into small pieces and filtered through nylon mesh into a sterile FALCON tube. To the tube, 30 ml of RPMI media was added. The cells were counted and seeded into 6-well tissue culture plates containing 3-4 ml/well, 5 x 10^6 cells/ml. Murine thymocytes are not viable after 48 hours.

Induction of VIAF Expression with Dexamethasone

To determine if VIAF is endogenously expressed in primary cells and tissues, such that the VIAF does not have to be over-expressed to study VIAF function, the level of VIAF expression was examined in thymocytes. Murine thymocytes were isolated as described above and stimulated with dexamethasone (10^{-7} M) or control media for the indicated amount of time. Dexamethasone is a glucocorticoid which induces apoptosis in human and murine thymocytes. The cells were subsequently lysed with RIPA-lysis buffer (Tris-HCl 50 mM, 150 mM, 1% NP40, 0.1% SDS, and 0.5.% deoxycholate) and proteins separated by SDS-PAGE and transferred onto a nitrocellulose membrane. VIAF was detected with a mixture of the two polyclonal anti-VIAF antibodies described in EXAMPLE 5.

Endogenous VIAF expression increased as a result of stimulating the thymocytes with dexamethasone after four hours (FIG. 10, compare lanes 1 and 2). Therefore, thymocytes contain sufficient levels of endogenous VIAF to study its role in apoptosis. These results indicate that VIAF may be necessary to induce and/or regulate this apoptotic process.

Apoptosis Inhibitors

To further investigate the role of VIAF in the apoptosis of murine thymocytes, the effect of several inhibitors of apoptosis was investigated.

Murine thymocytes were stimulated with or without dexamethasone (as described above) and co-incubated with the inhibitors (50 μM ZVAD-fmk; 50 μM lactacystin; 50 μM calpain inhibitor I; or media) as shown in FIG. 10. Cells were subsequently lysed with RIPA-lysis buffer (see above) and proteins separated by SDS-PAGE and transferred onto a nitrocellulose membrane. VIAF was detected with a mixture of the two polyclonal anti-VIAF antibodies described in EXAMPLE 5.

As shown in FIG. 10, expression of VIAF was induced by dexamethasone and this induction can be inhibited by ZVAD-fmk. However, VIAF expression was not inhibited by either lactacystin or calpain inhibitor I.

EXAMPLE 12

Cloning VIAF in Other Organisms

Having presented the nucleotide sequences of several homologs of VIAF cDNA and the amino acid sequences of the encoded proteins, this disclosure now also facilitates the identification

of DNA molecules, and thereby proteins, which are the VIAF homologs in the same or other species, for example polymorphisms in the same species or homologs in other species, such other primates. These other homologs can be derived from those sequences disclosed, but which vary in their precise nucleotide or amino acid sequence from those disclosed. Such variants may be obtained through a combination of standard molecular biology laboratory techniques and the nucleotide and amino acid sequence information disclosed herein.

The VIAF homologs in other organisms may be identified by using the VIAF sequences to design probes, for example an oligonucleotide or polynucleotide. Such probes can be used to screen a genomic or cDNA library from any organism using standard hybridization methods. In addition, primers or degenerate primers covering regions of VIAF thought to be important for its function (for example the C-terminal 128 amino acids of SEQ ID NO 2), can be designed for use in a PCR reaction to amplify VIAF homologs from a genomic or cDNA library.

EXAMPLE 13

Production of Sequence Variants of VIAF cDNAs and Proteins

SEQ ID NOs 1, 3, 5, and 7 show the nucleotide sequences of several VIAF homolog cDNAs, and the amino acid sequences of the VIAF proteins encoded by these cDNAs are shown in SEQ ID NOs 2, 4, 6, and 8, respectively. The distinctive functional characteristic of VIAF is its ability to modulate the anti-apoptotic and signaling properties of the IAP family. This activity of the VIAF protein may readily be determined using the assays described above, for example those described in EXAMPLES 7-10.

Having presented the nucleotide sequence of several VIAF cDNAs and the amino acid sequence of these proteins, this disclosure facilitates the creation of DNA molecules, and thereby proteins, which are derived from those disclosed but which vary in their precise nucleotide or amino acid sequence from those disclosed. Such variants may be obtained through a combination of standard molecular biology laboratory techniques and the nucleotide sequence information disclosed herein.

VIAF variants and fragments will retain the ability to modulate the anti-apoptotic and signaling properties of the IAP family. Since the region containing amino acids 7-21 of the human VIAF sequence is highly conserved between species (see FIG. 1), in particular embodiments these residues of VIAF ideally do not substantially diverge from the wild-type sequence shown in SEQ ID NOs 2, 4, 6, and 8. In other embodiments, the VIAF protein has less than 275 amino acids, for example less than 250, 240, or 237 amino acids. Alternatively, the VIAF protein may have 237, 236, 200, 150, or 100 amino acids. Other important residues include those domains noted in FIG. 2. Such domains include, but are not limited to: the SHC SH2 domain binding site, amino acids 48-52 of SEQ ID NO 2, ATP binding sites, amino acids 83-96 and 100-111 of SEQ ID NO 2, and

the OpIAP interaction domain, amino acids 111-239 of SEQ ID NO 2. In these regions, conservative substitutions will be better tolerated than non-conservative substitutions.

The indication of highly conserved regions in FIG. 1 provides further guidance in helping select residues that may be substituted or deleted. For example, referring to FIG. 1, the region containing amino acids 33-40 of the human VIAF sequence is not highly conserved among the five species shown. Therefore, alterations in the sequence in this region are predicted to have less of an effect on the function of the VIAF protein, than for example mutations in the region containing amino acids 7-21 of the human VIAF sequence. Variants and fragments may retain at least 60%, 70%, 80%, 85%, 95%, 98%, or greater sequence identity to the VIAF amino acid sequences disclosed herein, and in particular embodiments at least this much identity to SEQ ID NOs 2, 4, 6, and 8. Less identity is allowed, as long as the variant VIAF sequence maintains the functional activity of the VIAF protein as defined herein. Such activity can be readily determined using the assays disclosed herein.

The simplest modifications involve the substitution of one or more amino acid residues (for example 2, 5 or 10 residues) for amino acid residues having similar biochemical properties. These so-called conservative substitutions are likely to have minimal impact on the activity of the resultant protein. Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are conservative when it is desired to finely modulate the characteristics of the protein. Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val.

Amino acid substitutions are typically of single residues, for example 1, 2, 3, 4, 5, 10 or more substitutions; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. Obviously, the mutations that are made in the DNA encoding the protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those listed above, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in protein properties will be those in

which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. Such variants can be readily selected for additional testing by performing an assay (such as that shown in EXAMPLE 9) to determine if the variant can modulate anti-apoptotic signaling, for example inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example more than about 1.5 fold, for example more than about 2-fold, and to determine if co-expression of the variant with suboptimal quantities of XIAP will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli. The ability of the variant to modulate the anti-apoptotic signaling properties of the IAP family can be readily assayed using the methods described in EXAMPLE 10. The ability of VIAF to interact with OpIAP, XIAP, c-IAP1 and c-IAP2 can also be readily assayed as in EXAMPLES 7 and 8.

The effects of these amino acid substitutions or deletions or additions may be assessed for derivatives of the VIAF protein by the assays as described in the EXAMPLES above.

Variant DNA molecules include those created by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 15). By the use of such techniques, variants may be created which differ in minor ways from those disclosed. DNA molecules and nucleotide sequences which are derivatives of those specifically disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein which possesses the functional characteristics of the VIAF proteins, are comprehended by this disclosure.

Also within the scope of this disclosure are small DNA molecules which are derived from the disclosed DNA molecules. Such small DNA molecules include oligonucleotides suitable for use as hybridization probes or polymerase chain reaction (PCR) primers. As such, these small DNA molecules will comprise at least a segment of the VIAF cDNA molecules or the VIAF gene and, for the purposes of PCR, will comprise at least a 20, 30, 40 or 50 contiguous nucleotides of the VIAF cDNAs or genes from SEQ ID NOs. 1, 3, 5, 7, 9 or their complementary strands. It will be appreciated that such longer length nucleotide sequences will provide greater specificity in hybridization or PCR applications than shorter length sequences. Accordingly, superior results may be obtained using these longer stretches of consecutive nucleotides.

DNA molecules and nucleotide sequences which are derived from the disclosed DNA molecules as described above may also be defined as DNA sequences which hybridize under stringent conditions to the DNA sequences disclosed, or fragments thereof. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the

hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Chapters 9 and 11), herein incorporated by reference.

Specific hybridization refers to the binding, duplexing, or hybridizing of a molecule only or substantially only to a particular nucleotide sequence when that sequence is present in a complex mixture (e.g. total cellular DNA or RNA). Specific hybridization may also occur under conditions of varying stringency.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989 ch. 9 and 11), herein incorporated by reference. By way of illustration only, a hybridization experiment may be performed by hybridization of a DNA molecule (for example, a variant of the VIAF cDNA) to a target DNA molecule (for example, the VIAF cDNA) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern, *J. Mol. Biol.* 98:503, 1975), a technique well known in the art and described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Hybridization with a target probe labeled with [^{32}P]-dCTP is generally carried out in a solution of high ionic strength such as 6xSSC at a temperature that is 20-25°C below the melting temperature, T_m, described below. For such Southern hybridization experiments where the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to 10^9 CPM/μg or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization. The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal.

The term T_m represents the temperature above which, under the prevailing ionic conditions, the radiolabeled probe molecule will not hybridize to its target DNA molecule. The T_m of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy, *Proc. Natl. Acad. Sci. USA* 48:1390, 1962): $T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%\text{ formamide}) - (600/l)$; where l = the length of the hybrid in base pairs.

This equation is valid for concentrations of Na^+ in the range of 0.01 M to 0.4 M, and it is less accurate for calculations of T_m in solutions of higher [Na^+]. The equation is also primarily valid for DNAs whose G+C content is in the range of 30% to 75%, and it applies to hybrids greater than 100 nucleotides in length (the behavior of oligonucleotide probes is described in detail in Ch. 11 of Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Thus, by way of example, for a 150 base pair DNA probe derived from the open reading frame of the VIAF cDNA (with a hypothetical %GC = 45%), a calculation of hybridization conditions required to give particular stringencies may be made as follows: For this example, it is assumed that the filter will be washed in 0.3 xSSC solution following hybridization, thereby: [Na^+] = 0.045 M; %GC = 45%; Formamide concentration = 0; l = 150 base pairs; T_m=81.5 - 16.6($\log_{10}$[Na^+]) + (0.41 x 45) - (600/150); and so T_m = 74.4°C.

The T_m of double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., *J. Mol. Biol.* 81:123, 1973). Therefore, for this given example, washing the filter in 0.3 xSSC at 59.4-64.4°C will produce a stringency of hybridization equivalent to 90%; that is, DNA molecules with more than 10% sequence variation relative to the target VIAF cDNA will not hybridize. Alternatively, washing the hybridized filter in 0.3 xSSC at a temperature of 65.4-68.4°C will yield a hybridization stringency of 94%; that is, DNA molecules with more than 6% sequence variation relative to the target VIAF cDNA molecule will not hybridize. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

Examples of stringent conditions are those under which DNA molecules with more than 25%, 15%, 10%, 6% or 2% sequence variation (also termed "mismatch") will not hybridize. Stringent conditions are sequence dependent and are different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be no more than about 5°C lower than the thermal melting point T_m for the specific sequence at a defined ionic strength and pH. An example of stringent conditions is a salt concentration of at least about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and a temperature of at least about 30°C for short probes (e.g. 10 to 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For example, conditions of 5X SSPE (750 mM NaCl, 50 mM Na Phosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C are suitable for allele-specific probe hybridizations.

A perfectly matched probe has a sequence perfectly complementary to a particular target sequence. The test probe is typically perfectly complementary to a portion (subsequence) of the target sequence. The term "mismatch probe" refers to probes whose sequence is deliberately selected not to be perfectly complementary to a particular target sequence.

Transcription levels can be quantitated absolutely or relatively. Absolute quantitation can be accomplished by inclusion of known concentrations of one or more target nucleic acids (for example control nucleic acids such as Bio B or with a known amount the target nucleic acids themselves) and referencing the hybridization intensity of unknowns with the known target nucleic acids (for example by generation of a standard curve).

The degeneracy of the genetic code further widens the scope of the present disclosure as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. For example, the sixth amino acid residue of the human VIAF protein is alanine. This is encoded in the VIAF cDNA by the nucleotide codon triplet GCA. Because of the degeneracy of the genetic code, three other nucleotide codon triplets, GCT, GCG and GCC, also code for alanine. Thus, the nucleotide sequence of the VIAF cDNA could be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the cDNA molecules disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. DNA sequences which do not hybridize under stringent conditions to the cDNA sequences disclosed by virtue of sequence variation based on the degeneracy of the genetic code are herein also comprehended by this disclosure.

One skilled in the art will recognize that the DNA mutagenesis techniques described above may be used not only to produce variant DNA molecules, but will also facilitate the production of proteins which differ in certain structural aspects from the VIAF proteins, yet which proteins are clearly derivative of this protein and which maintain the essential characteristics of the VIAF protein. Newly derived proteins may also be selected in order to obtain variations on the characteristic of the VIAF protein, as described herein. Such derivatives include those with variations in amino acid sequence including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

The *VIAF* gene, *VIAF* cDNA, DNA molecules derived therefrom and the proteins encoded by these cDNAs and derivative DNA molecules may be utilized in aspects of both the study of VIAF and for diagnostic and therapeutic applications related to VIAF. Utilities disclosed herein include, but are not limited to, the modulation of anti-apoptotic and signaling properties of the IAP family. Those skilled in the art will recognize that the utilities herein described are not limited to

the specific experimental modes and materials presented and will appreciate the wider potential utility of this disclosure.

EXAMPLE 14

Recombinant Expression of VIAF

With the provision of several homologus VIAF cDNA sequences (SEQ ID NOs: 1, 3, 5, 7, and 9), the expression and purification of any VIAF protein homolog by standard laboratory techniques is now enabled. The purified protein may be used for functional analyses, antibody production, diagnosis, and therapy in a subject. Furthermore, the DNA sequence of the VIAF cDNA can be manipulated in studies to understand the expression of the gene and the function of its product. Mutant forms of VIAF may be isolated based upon information contained herein, and may be studied in order to detect alteration in expression patterns in terms of relative quantities, tissue specificity and functional properties of the encoded mutant VIAF protein. Partial or full-length cDNA sequences, which encode for the subject protein, may be ligated into bacterial expression vectors. Methods for expressing large amounts of protein from a cloned gene introduced into *E. coli* may be utilized for the purification, localization and functional analysis of proteins. For example, fusion proteins consisting of amino terminal peptides encoded by a portion of the *E. coli* lacZ or trpE gene linked to VIAF protein may be used to prepare polyclonal and monoclonal antibodies against this protein. Thereafter, these antibodies may be used to purify proteins by immunoaffinity chromatography, in diagnostic assays to quantitate the levels of protein and to localize proteins in tissues and individual cells by immunofluorescence.

Intact native protein may also be produced in *E. coli* in large amounts for functional studies. Methods and plasmid vectors for producing fusion proteins and intact native proteins in bacteria are described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, chapter 17, herein incorporated by reference). Such fusion proteins may be made in large amounts, are easy to purify, and can be used to elicit antibody response. Native proteins can be produced in bacteria by placing a strong, regulated promoter and an efficient ribosome binding site upstream of the cloned gene. If low levels of protein are produced, additional steps may be taken to increase protein production; if high levels of protein are produced, purification is relatively easy. Suitable methods are presented in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) and are well known in the art. Often, proteins expressed at high levels are found in insoluble inclusion bodies. Methods for extracting proteins from these aggregates are described by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Chapter 17).

Vector systems suitable for the expression of lacZ fusion genes include the pUR series of vectors (Ruther and Muller-Hill, 1983, *EMBO J.* 2:1791), pEX1-3 (Stanley and Luzio, 1984, *EMBO J.* 3:1429) and pMR100 (Gray et al., 1982, *Proc. Natl. Acad. Sci. USA* 79:6598). Vectors

suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg, 1981, *Nature* 292:128), pKK177-3 (Amann and Brosius, 1985, *Gene* 40:183) and pET-3 (Studiar and Moffatt, 1986, *J. Mol. Biol.* 189:113). VIAF fusion proteins may be isolated from protein gels, lyophilized, ground into a powder and used as an antigen. The DNA sequence can also be transferred to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke et al., 1987, *Science* 236:806-12). These vectors may then be introduced into a variety of hosts including somatic cells, and simple or complex organisms, such as bacteria, fungi (Timberlake and Marshall, 1989, *Science* 244:1313-7), invertebrates, plants (Gasser and Fraley, 1989, Science 244:1293), and mammals (Pursel et al., 1989, *Science* 244:1281-8), which cell or organisms are rendered transgenic by the introduction of the heterologous VIAF cDNA.

For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus SV40, promoter in the pSV2 vector (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6), and introduced into cells, such as monkey COS-1 cells (Gluzman, 1981, *Cell* 23:175-82), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg, 1982, *J. Mol. Appl. Genet.* 1:327-41) and mycophoenolic acid (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6).

DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR.

The cDNA sequence (or portions derived from it) or a mini gene (a cDNA with an intron and its own promoter) may be introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the transcription of the cDNA eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the SV40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV40 are readily available (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6; Gorman *et al.*, 1982, *Proc. Natl. Acad. Sci USA* 78:6777-81). The level of expression of the cDNA can be manipulated with this type of vector, either by using promoters that have different activities (for example, the baculovirus pAC373 can express cDNAs at high levels in *S. frugiperda* cells (Summers and Smith, 1985, Genetically Altered Viruses and the Environment, Fields *et al.* (Eds.) 22:319-328, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.) or by using vectors that contain promoters amenable to modulation, for example, the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee *et al.*, 1982,

Nature 294:228). The expression of the cDNA can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

In addition, some vectors contain selectable markers such as the *gpt* (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6) or *neo* (Southern and Berg, 1982, *J. Mol. Appl. Genet.* 1:327-41) bacterial genes. These selectable markers permit selection of transfected cells that exhibit stable, long-term expression of the vectors (and therefore the cDNA). The vectors can be maintained in the cells as episomal, freely replicating entities by using regulatory elements of viruses such as papilloma (Sarver *et al.*, 1981, *Mol. Cell Biol.* 1:486) or Epstein-Barr (Sugden *et al.*, 1985, *Mol. Cell Biol.* 5:410). Alternatively, one can also produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the cDNA as well) to create cell lines that can produce high levels of the gene product (Alt *et al.*, 1978, *J. Biol. Chem.* 253:1357).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells, is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, 1973, *Virology* 52:466) or strontium phosphate (Brash *et al.*, 1987, *Mol. Cell Biol.* 7:2013), electroporation (Neumann *et al.*, 1982, *EMBO J.* 1:841), lipofection (Felgner et al., 1987, *Proc. Natl. Acad. Sci USA* 84:7413), DEAE dextran (McCuthan *et al.*, 1968, *J. Natl. Cancer Inst.* 41:351), microinjection (Mueller *et al.*, 1978, *Cell* 15:579), protoplast fusion (Schafner, 1980, *Proc. Natl. Acad. Sci. USA* 77:2163-7), or pellet guns (Klein *et al.*, 1987, *Nature* 327:70). Alternatively, the cDNA can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein *et al.*, 1985, *Gen. Engrg.* 7:235), adenoviruses (Ahmad *et al.*, 1986, *J. Virol.* 57:267), or Herpes virus (Spaete et al., 1982, *Cell* 30:295).

These eukaryotic expression systems can be used for studies of the VIAF gene and mutant forms of this gene, the VIAF protein and mutant forms of this protein. Such uses include, for example, the identification of regulatory elements located in the 5' region of the VIAF gene on genomic clones that can be isolated from genomic DNA libraries, such as human or mouse libraries, using the information contained herein. The eukaryotic expression systems may also be used to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring or artificially produced mutant proteins. Naturally occurring mutant proteins may exist in a variety of cancers or diseases, while artificially produced mutant proteins can be designed by site directed mutagenesis as described herein. These latter studies may probe the function of any desired amino acid residue in the protein by mutating the nucleotide coding for that amino acid.

Using the above techniques, the expression vectors containing the VIAF gene or cDNA sequence or fragments or variants or mutants thereof can be introduced into human cells,

mammalian cells from other species or non-mammalian cells as desired. The choice of cell is determined by the purpose of the treatment. For example, monkey COS cells (Gluzman, 1981, *Cell* 23:175-82) that produce high levels of the SV40 T antigen and permit the replication of vectors containing the SV40 origin of replication may be used. Similarly, Chinese hamster ovary (CHO), mouse NIH 3T3 fibroblasts or human fibroblasts or lymphoblasts may be used.

One method that can be used to express the VIAF polypeptide from the cloned VIAF cDNA sequence in mammalian cells is to use the cloning vector pXTI (Stratagene). This vector contains the Long Terminal Repeats (LTRs) and a portion of the GAG gene from Moloney Murine Leukemia Virus. The position of the viral LTRs allows highly efficient, stable transfection of the region within the LTRs. The vector also contains the Herpes Simplex Thymidine Kinase promoter (TK), active in embryonal cells and in a wide variety of tissues in mice, and a selectable neomycin gene conferring G418 resistance. Two unique restriction sites, BglII and XhoI, are directly downstream from the TK promoter. VIAF cDNA, including the entire open reading frame for the VIAF protein and the 3' untranslated region of the cDNA is cloned into one of the two unique restriction sites downstream from the promoter.

The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. Positive transfectants are selected after growing the transfected cells in 600 μg/ml G418 (Sigma, St. Louis, MO). The protein is released into the supernatant and may be purified by standard immunoaffinity chromatography techniques using antibodies raised against the VIAF protein (see Examples 5 and 15).

Expression of VIAF protein in eukaryotic cells can be used as a source of proteins to raise antibodies. The VIAF protein may be extracted following release of the protein into the supernatant as described above, or, the cDNA sequence may be incorporated into a eukaryotic expression vector and expressed as a chimeric protein with, for example, β-globin. Antibody to β-globin is thereafter used to purify the chimeric protein. Corresponding protease cleavage sites engineered between the β-globin gene and the cDNA are then used to separate the two polypeptide fragments from one another after translation. One useful expression vector for generating β-globin chimeric proteins is pSG5 (Stratagene). This vector encodes rabbit β-globin.

The recombinant vector then contains the selected DNA of the DNA sequnces disclosed herein for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that the VIAF polypeptide can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be specifically selected from the group consisting of the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and

simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

The host cell, which may be transfected with the vector disclosed herein, may be selected from the group consisting of: *E. coli*, *Pseudomonas*, *B. subtilis*, *B. stearothermophilus* or other bacilli; other bacteria; yeast; fungi; plant; insect; mouse or other animal; or human tissue cells.

It is appreciated that for mutant or variant VIAF DNA sequences, similar systems are employed to express and produce the mutant or variant product.

EXAMPLE 15

Production of Anti-VIAF Antibodies

Monoclonal or polyclonal antibodies may be produced to any of the VIAF proteins herein disclosed, or variants, fragments and mutant forms thereof. Optimally, antibodies raised against VIAF would specifically detect the VIAF protein. That is, such antibodies would recognize and bind the protein and would not substantially recognize or bind to other proteins found in human or other cells. The determination that an antibody specifically detects the VIAF protein is made by any one of a number of standard immunoassay methods; for instance, the Western blotting technique (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). To determine that a given antibody preparation (such as one produced in a mouse against VIAF) specifically detects the VIAF protein by Western blotting, total cellular protein is extracted from cells (for example, lymphocytes) and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. The proteins are then transferred to a membrane (for example, nitrocellulose) by Western blotting, and the antibody preparation is incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies is detected by the use of an anti-mouse antibody conjugated to an enzyme such as alkaline phosphatase; application of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a dense blue compound by immuno-localized alkaline phosphatase.

Antibodies which specifically detect the VIAF protein will, by this technique, be shown to bind to the VIAF protein band (which will be localized at a given position on the gel determined by its molecular weight). Non-specific binding of the antibody to other proteins may occur and may be detectable as a weak signal on the Western blot. The non-specific nature of this binding will be recognized by one skilled in the art by the weak signal obtained on the Western blot relative to the strong primary signal arising from the specific antibody-VIAF protein binding.

Substantially pure VIAF protein suitable for use as an immunogen is isolated as already described. Concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. Monoclonal or polyclonal antibody to the protein can then be prepared.

Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes of the VIAF protein can be identified, isolated and prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495, 1975) or derivative methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein over a period of a few weeks. The mouse is then sacrificed, and the antibody-producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Enzymol.* 70:419, 1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Harlow and Lane (Antibodies: A Laboratory Manual. 1988, Cold Spring Harbor Laboratory, New York). In addition, protocols for producing humanized forms of monoclonal antibodies (for therapeutic applications) and fragments of monoclonal antibodies are known in the art.

Polyclonal Antibody Production by Immunization

Polyclonal antiserum containing antibodies to heterogeneous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein (for example see EXAMPLES 6 and 14), which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis et al. (*J. Clin. Endocrinol. Metab.* 33:988-91, 1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony et al. (In: *Handbook of Experimental Immunology*, Wier, D. (ed.). Chapter 19. Blackwell. 1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher (*Manual of Clinical Immunology*, Chapter 42. 1980).

Antibodies Raised against Synthetic Peptides

Another approach to raising antibodies against VIAF is to use synthetic peptides synthesized on a commercially available peptide synthesizer based upon the predicted amino acid sequence of the VIAF protein, for example SEQ ID NOs 2, 4, 6, 8 and 10. The chemical synthesis described in EXAMPLE 23 for example may be used to generate a synthetic VIAF protein.

Antibodies Raised by Injection of VIAF cDNA

Antibodies may be raised against the VIAF protein by subcutaneous injection of a DNA vector which expresses the VIAF protein into laboratory animals, such as mice. Delivery of the recombinant vector into the animals may be achieved using a hand-held form of the Biolistic system (Sanford *et al.*, *Particulate Sci. Technol.* 5:27-37, 1987) as described by Tang *et al.* (*Nature* 356:152-4, 1992). Expression vectors suitable for this purpose may include those which express the VIAF cDNA under the transcriptional control of either the human β-actin promoter or the cytomegalovirus (CMV) promoter.

Antibody preparations prepared according to these protocols are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample.

Labeled Antibodies

Antibodies disclosed herein can be conjugated with various labels for their direct detection (see Chapter 9, Harlow and Lane, *Antibodies: A Laboratory Manual.* 1988). The label, which may include, but is not limited to, a radiolabel, enzyme, fluorescent probe, or biotin, is chosen based on the method of detection available to the user.

Antibodies can be radiolabeled with iodine (^{125}I), which yields low-energy gamma and X-ray radiation. Briefly, 10 μg of protein in 25 μl of 0.5 M sodium phosphate (pH 7.50 is placed in a 1.5 ml conical tube. To this, 500 μC of $Na^{125}I$, and 25 μl of 2 mg/ml chloramine T is added and incubated for 60 seconds at room temperature. To stop the reaction, 50μl of chloramine T stop buffer is added (2.4 mg/ml sodium metabisulfite, 10 mg/ml tyrosine, 10% glycerol, 0.1% xylene cyanol in PBS). The iodinated antibody is separated from the iodotyrosine on a gel filtration column. Antibodies disclosed herein can also be labeled with biotin, with enzymes such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) or with fluorescent dyes. The method of producing these conjugates is determined by the reactive group on the label added.

EXAMPLE 16

Diagnostic Methods

An embodiment disclosed herein is a method for screening a subject to determine if the subject carries a mutant or variant VIAF gene, for example having a heterozygous or homozygous nucleotide change, or insertions or deletions of the VIAF gene, including partial or complete deletion of the gene. One major application of the VIAF sequence information presented herein is in the area of genetic testing for predisposition to disease, such as: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; 6-12 leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes, owing to a VIAF deletion or mutation. The gene sequence of the VIAF genes, including intron-exon boundaries is also useful in such diagnostic methods. The method consists of providing a biological sample obtained from the subject, in which the sample includes DNA or RNA, and providing an assay for detecting in the biological sample the presence of a mutant VIAF gene, a mutant VIAF RNA, a homozygously or heterozygously deleted VIAF gene, or the absence, through deletion, of the VIAF gene and corresponding RNA. Suitable biological samples include samples obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, fine needle aspirate specimen, amniocentesis samples and autopsy material. The detection in the biological sample may be performed by a number of methodologies, as outlined below.

The foregoing assay may be assembled in the form of a diagnostic kit and may include, for example: hybridization with oligonucleotides; PCR amplification of the gene or a part thereof using oligonucleotide primers; RT-PCR amplification of the RNA or a part thereof using oligonucleotide primers; or direct sequencing of the VIAF gene of the subject's genome using oligonucleotide primers. The efficiency of these molecular genetic methods should permit a rapid classification of patients affected by mutations, deletions or variants of the VIAF gene.

One embodiment of such detection techniques is the polymerase chain reaction amplification of reverse transcribed RNA (RT-PCR) of RNA isolated from cells (for example lymphocytes) followed by direct DNA sequence determination of the products. The presence of one or more nucleotide differences between the obtained sequence and the cDNA sequences, and especially, differences in the ORF portion of the nucleotide sequence are taken as indicative of a potential VIAF gene mutation.

Alternatively, DNA extracted from lymphocytes or other cells may be used directly for amplification. The direct amplification from genomic DNA would be appropriate for analysis of the entire VIAF gene including regulatory sequences located upstream and downstream from the open reading frame. Recent reviews of direct DNA diagnosis have been presented by Caskey (*Science* 236:1223-1228, 1989) and by Landegren et al. (*Science* 242:229-37, 1989).

Further studies of VIAF genes isolated from subjects may reveal particular mutations, deletions, or variants which occur at a high frequency within this population of individuals. In this case, rather than sequencing the entire VIAF gene, it is possible to design DNA diagnostic methods to specifically detect the most common VIAF mutations, deletions, or variants.

The detection of specific DNA mutations may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 51:257-61), direct DNA sequencing (Church and Gilbert, 1984, *Proc. Natl. Acad. Sci. USA*. 81:1991-5), the use of restriction enzymes (Flavell et al., 1978, *Cell* 15:25; Geever et al., 1981, *Proc. Natl. Acad. Sci USA* 78:5081), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis, 1986, *Cold Spring Harbor Symp. Quant. Biol.* 51:275-284), RNase protection (Myers et al., 1985, *Science* 230:1242), chemical cleavage (Cotton et al., 1985, *Proc. Natl. Acad. Sci. USA* 85:4397-401), and the ligase-mediated detection procedure (Landegren et al., 1988, *Science* 241:1077).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labeled radioactively with isotopes (such as ^{32}P) or non-radioactively, with tags such as biotin (Ward and Langer et al., 1981. *Proc. Natl. Acad. Sci. USA* 78:6633-57), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence of these specific sequences are visualized by methods such as autoradiography or fluorometric (Landegren et al., 1989, *Science* 242:229-37) or colorimetric reactions (Gebeyehu et al., 1987, *Nucleic Acids Res.* 15:4513-34). The absence of hybridization would indicate a mutation in the particular region of the gene, or a deleted VIAF gene.

Sequence variations between normal and mutant forms of the VIAF gene may also be revealed by the direct DNA sequencing method of Church and Gilbert (*Proc. Natl. Acad. Sci. USA* 81:1991-5, 1988). Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR (Wrichnik et al., 1987, *Nucleic Acids Res.* 15:529-42; Wong et al., 1987, *Nature* 330:384-6; Stoflet et al., 1988, *Science* 239:491-4). In this approach, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence alterations may occasionally generate fortuitous restriction enzyme recognition sites or may eliminate existing restriction sites. Changes in restriction sites are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization (Southern, 1975, *J. Mol. Biol.* 98:503). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme;

fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high-resolution gel electrophoresis. For example, a PCR product with small deletions is clearly distinguishable from a normal sequence on an 8% non-denaturing polyacrylamide gel (WO 91/10734; Nagamine et al., 1989, *Am. J. Hum. Genet.* 45:337-9). DNA fragments of different sequence compositions may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperatures (Myers et al., 1985, *Science* 230:1242). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length in a PCR. For example, an invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.

In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments can be visualized by methods where individual DNA samples are not immobilized on membranes. The probe and target sequences may be both in solution, or the probe sequence may be immobilized (Saiki *et al.*, 1989, *Proc. Nat. Acad. Sci. USA* 86:6230-4). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving calorigenic reactions and fluorometry involved fluorogenic reactions, may be used to identify specific individual genotypes.

If more than one mutation is frequently encountered in the VIAF gene, a system capable of detecting such multiple mutations is desirable. For example, a PCR with multiple, specific oligonucleotide primers and hybridization probes may be used to identify all possible mutations at the same time (Chamberlain *et al.*, 1988, *Nucl. Acids Res.* 16:1141-55). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki *et al.*, 1989, *Proc. Nat. Acad. Sci. USA* 86:6230-4).

EXAMPLE 17

Quantitation of VIAF Proteins

An alternative method of diagnosing a VIAF gene deletion, variant, or other mutation is to quantitate the level of VIAF protein in the cells of a subject. This diagnostic tool is useful for detecting reduced levels of the VIAF protein which result from, for example, mutations in the promoter regions of the VIAF gene or mutations within the coding region of the gene which produced truncated, non-functional polypeptides, as well as from deletions of the entire VIAF gene.

These diagnostic methods, in addition to those described in EXAMPLE 16, provide an enhanced ability to diagnose susceptibility to diseases caused by mutation or deletion of these genes.

The determination of reduced VIAF protein levels would be an alternative or supplemental approach to the direct determination of VIAF gene deletion or mutation status by the methods outlined above in EXAMPLE 16. The availability of antibodies specific to the VIAF protein (for example those described in Examples 5 and 15) will facilitate the quantitation of cellular VIAF protein by one of a number of immunoassay methods which are well known in the art and are presented in Harlow and Lane (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York. 1988).

Such assays permit both the detection of VIAF proteins in a biological sample and the quantitation of such proteins. Typical methods involve: providing a biological sample of the subject in which the sample contains cellular proteins, and providing an immunoassay for quantitating the level of VIAF protein in the biological sample. This can be achieved by combining the biological sample with a VIAF specific binding agent, such as an anti-VIAF antibody (such as monoclonal or polyclonal antibodies), so that complexes form between the binding agent and the VIAF protein present in the sample, and then detecting or quantitating such complexes.

In particular forms, these assays may be performed with the VIAF specific binding agent immobilized on a support surface, such as in the wells of a microtiter plate or on a column. The biological sample is then introduced onto the support surface and allowed to interact with the specific binding agent so as to form complexes. Excess biological sample is then removed by washing, and the complexes are detected with a reagent, such as a second anti-VIAF protein antibody that is conjugated with a detectable marker.

In an alternative assay, the cellular proteins are isolated and subjected to SDS-PAGE followed by Western blotting, for example as described in EXAMPLE 6. After resolving the proteins, the proteins are transferred to a membrane, which is probed with specific binding agents that recognize VIAF. The proteins are detected, for example with HRP-conjugated secondary antibodies, and quantitated.

In yet another assay, the level of VIAF protein in cells is analyzed using microscopy. Using specific binding agents which recognize VIAF, samples can be analyzed for the presence of VIAF proteins. For example, frozen biopsied tissue sections are thawed at room temperature and fixed with acetone at -200°C for five minutes. Slides are washed twice in cold PBS for five minutes each, then air-dried. Sections are covered with 20-30 μl of antibody solution (15-45 μg/ml) (diluted in PBS, 2% BSA at 15-50 μg/ml) and incubated at RT in a humidified chamber for 30 minutes. Slides are washed three times with cold PBS five minutes each, allowed to air-dry briefly (5 minutes) before applying 20-30 μl of the second antibody solution (diluted in PBS, 2% BSA at 15-50 μg/ml) and incubated at room temperature in humidified chamber for 30 minutes. The label on the second antibody may contain a fluorescent probe, enzyme, radiolabel, biotin, or

other detectable marker. The slides are washed three times with cold PBS 5 minutes each then quickly dipped in distilled water, air-dried, and mounted with PBS containing 30% glycerol. Slides can be stored at 4°C prior to viewing.

For samples prepared for electron microscopy (versus light microscopy), the second antibody is conjugated to gold particles. Tissue is fixed and embedded with epoxy plastics, then cut into very thin sections (~1-2 μm). The specimen is then applied to a metal grid, which is then incubated in the primary anti-VIAF antibody, washed in a buffer containing BSA, then incubated in a secondary antibody conjugated to gold particles (usually 5-20 nm). These gold particles are visualized using electron microscopy methods.

For the purposes of quantitating the VIAF proteins, a biological sample of the subject, which sample includes cellular proteins, is required. Such a biological sample may be obtained from body cells, such as those present in which expression of the protein has been detected. As shown in FIG. 3, for example, VIAF could be analyzed in cells isolated from the testis, ovary or prostate, but its expression in peripheral blood leukocytes is clearly the most accessible and convenient source from which specimens can be obtained. Specimens can be obtained from peripheral blood, urine, saliva, tissue biopsy, amniocentesis samples, surgical specimens, fine needle aspirates, and autopsy material, particularly cancer cells. Quantitation of VIAF proteins would be made by immunoassay and compared to levels of the protein found in non-VIAF expressing human cells or to the level of VIAF in healthy cells (cells of the same origin that are not neoplastic). A significant (for example 50% or greater) reduction in the amount of VIAF protein in the cells of a subject compared to the amount of VIAF protein found in non-VIAF expressing cells or that found in normal cells, would be taken as an indication that the subject may have deletions or mutations in the VIAF gene locus.

EXAMPLE 18

Gene Therapy

A new gene therapy approach for subjects suffering from VIAF gene deletions or mutations is now made possible by the present disclosure. Essentially, cells may be removed from a subject having deletions or mutations of the VIAF gene, and then transfected with an expression vector containing VIAF cDNA. These transfected cells will thereby produce functional VIAF protein and can be reintroduced into the subject.

The scientific and medical procedures required for human cell transfection are now routine procedures. The provision herein of VIAF cDNAs now allows the development of human gene therapy based upon these procedures. Immunotherapy of melanoma patients using genetically engineered tumor-infiltrating lymphocytes (TILs) has been reported by Rosenberg *et al.* (*N. Engl. J. Med.* 323:570-8, 1990). In that study, a retrovirus vector was used to introduce a gene for

neomycin resistance into TILs. A similar approach may be used to introduce the VIAF cDNA into patients affected by VIAF deletions or mutations.

In some embodiments, a method of treating tumors which underexpress VIAF, or in which greater VIAF expression is desired, is disclosed. These methods can be accomplished by introducing a gene coding for VIAF into a subject. A general strategy for transferring genes into donor cells is disclosed in U.S. Patent No. 5,529,774, incorporated by reference. Generally, a gene encoding a protein having therapeutically desired effects is cloned into a viral expression vector, and that vector is then introduced into the target organism. The virus infects the cells, and produces the protein sequence *in vivo*, where it has its desired therapeutic effect. See, for example, Zabner *et al.* (*Cell* 75:207-16, 1993).

In some of the foregoing examples, it may only be necessary to introduce the genetic or protein elements into certain cells or tissues. For example, in the case of benign nevi and psoriasis, introducing them into only the skin may be sufficient. However, in some instances (i.e. tumors and polycythemia inflammatory fibrosis), it may be more therapeutically effective and simple to treat all of the patients cells, or more broadly disseminate the vector, for example by intravascular administration.

The nucleic acid sequence encoding at least one therapeutic agent is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, the gene's native promoter, retroviral LTR promoter, or adenoviral promoters, such as the adenoviral major late promoter; the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMTV promoter; the metallothionein promoter; heat shock promoters; the albumin promoter; the histone promoter; the α-actin promoter; TK promoters; B19 parvovirus promoters; and the ApoAI promoter. However the scope of the disclosure is not limited to specific foreign genes or promoters.

The recombinant nucleic acid can be administered to the subject by any method which allows the recombinant nucleic acid to reach the appropriate cells. These methods include injection, infusion, deposition, implantation, or topical administration. Injections can be intradermal or subcutaneous. The recombinant nucleic acid can be delivered as part of a viral vector, such as avipox viruses, recombinant vaccinia virus, replication-deficient adenovirus strains or poliovirus, or as a non-infectious form such as naked DNA or liposome encapsulated DNA.

EXAMPLE 19

Viral Vectors for Gene Therapy

Adenoviral vectors may include essentially the complete adenoviral genome (Shenk *et al.*, *Curr. Top. Microbiol. Immunol.* 111:1-39, 1984). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted. In one embodiment, the vector includes an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral

encapsidation signal; a DNA sequence encoding a therapeutic agent; and a promoter for expressing the DNA sequence encoding a therapeutic agent. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not necessarily free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins transcribed by the adenoviral major late promoter. In another embodiment, the vector may be an adeno-associated virus (AAV) such as described in U.S. Patent No. 4,797,368 (Carter *et al.*) and in McLaughlin et al. (*J. Virol.* 62:1963-73, 1988) and AAV type 4 (Chiorini *et al. J. Virol.* 71:6823-33, 1997) and AAV type 5 (Chiorini *et al. J. Virol.* 73:1309-19, 1999)

Such a vector may be constructed according to standard techniques, using a shuttle plasmid which contains, beginning at the 5' end, an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a tripartite leader sequence, a multiple cloning site (which may be as herein described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The DNA segment serves as a substrate for homologous recombination with a modified or mutated adenovirus, and may encompass, for example, a segment of the adenovirus 5' genome no longer than from base 3329 to base 6246. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. A desired DNA sequence encoding a therapeutic agent may be inserted into the multiple cloning site of the plasmid.

The plasmid may be used to produce an adenoviral vector by homologous recombination with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell line, such as 293 cells, by $CaPO_4$ precipitation. The homologous recombination produces a recombinant adenoviral vector which includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the adenovirus may be constructed by using a yeast artificial chromosome (or YAC) containing an adenoviral genome according to the method described in Ketner et al. (*Proc. Natl. Acad. Sci. USA*, 91:6186-90, 1994), in conjunction with the teachings contained herein. In this embodiment, the adenovirus yeast artificial chromosome is produced by homologous recombination *in vivo* between adenoviral DNA and yeast artificial chromosome plasmid vectors carrying segments of the adenoviral left and right genomic termini. A DNA sequence encoding a therapeutic agent then may be cloned into the adenoviral DNA. The modified adenoviral genome then is excised from the adenovirus yeast artificial chromosome in order to be used to generate adenoviral vector particles as hereinabove described.

The adenoviral particles are administered in an amount effective to produce a therapeutic effect in a subject. The exact dosage of adenoviral particles to be administered is dependent upon a variety of factors, including the age, weight, and sex of the subject to be treated, and the nature and extent of the disease or disorder to be treated. The adenoviral particles may be administered as part of a preparation having a titer of adenoviral particles of at least 1 x 10^{10} pfu/ml, and in general not exceeding 2 x 10^{11} pfu/ml. The adenoviral particles may be administered in combination with a pharmaceutically acceptable carrier in a volume up to 10 ml. The pharmaceutically acceptable carrier may be, for example, a liquid carrier such as a saline solution, protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ), or Polybrene (Sigma Chemical) as well as those described in EXAMPLE 24.

In another embodiment, the viral vector is a retroviral vector. Retroviruses have been considered for experiments in gene therapy because they have a high efficiency of infection and stable integration and expression (Orkin *et al.*, 1988, *Prog. Med. Genet.*7:130-42). The full length VIAF gene or cDNA can be cloned into a retroviral vector and driven from either its endogenous promoter or from the retroviral LTR (long terminal repeat). Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. The vector is generally a replication defective retrovirus particle.

Retroviral vectors are useful as agents to effect retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

Other viral transfection systems may also be utilized for this type of approach, including Vaccinia virus (Moss *et al.*, 1987, *Annu. Rev. Immunol.* 5:305-24), Bovine Papilloma virus (Rasmussen *et al.*, 1987, *Methods Enzymol.* 139:642-54) or members of the herpes virus group such as Epstein-Barr virus (Margolskee *et al.*, 1988, *Mol. Cell. Biol.* 8:2837-47). Recent developments in gene therapy techniques include the use of RNA-DNA hybrid oligonucleotides, as described by Cole-Strauss *et al.* (*Science* 273:1386-9, 1996). This technique can allow for site-specific integration of cloned sequences, permitting accurately targeted gene replacement.

New genes may be incorporated into proviral backbones in several general ways. In the most straightforward constructions, the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more

than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter. Alternatively, two genes may be expressed from a single promoter by the use of an Internal Ribosome Entry Site.

EXAMPLE 20

Two Step Assay to Detect the Presence of VIAF Gene in a Sample

A tissue sample from a subject is processed according to the method disclosed by Antonarakis *et al.* (*New Eng. J. Med.* 313:842-848, 1985), separated through a 1% agarose gel and transferred to a nylon membrane for Southern blot analysis. Membranes are UV cross linked at 150 mJ using a GS Gene Linker (Bio-Rad). A VIAF probe (for example, SEQ ID No: 1) is subcloned into pTZ18U. The phagemids are transformed into *E. coli* MV 1190 infected with M13KO7 helper phage (Bio-Rad, Richmond, Calif.). Single stranded DNA is isolated according to standard procedures (Sambrook, *et al. Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Blots are prehybridized for 15-30 minutes at 65°C in 7% sodium dodecyl sulfate (SDS) in 0.5 M $NaPO_4$. The methods follow those described by Nguyen *et al.* (*BioTechniques* 13:116-23, 1992). The blots are hybridized overnight at 65°C in 7% SDS, 0.5 M $NaPO_4$ with 25-50 ng/ml single stranded probe DNA. Post-hybridization washes consist of two 30 minute washes in 5% SDS, 40 mM $NaPO_4$ at 65°C, followed by two 30-minute washes in 1% SDS, 40 mM $NaPO_4$ at 65°C.

The blots are subsequently rinsed with phosphate buffered saline (pH 6.8) for five minutes at RT and incubated with 0.2% casein in PBS for five minutes. The blots are then preincubated for 5-10 minutes in a shaking water bath at 45°C with hybridization buffer consisting of 6 M urea, 0.3 M NaCl, and 5X Denhardt's solution (see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). The buffer is removed and replaced with 50-75 $\mu l/cm^2$ fresh hybridization buffer plus 2.5 nM of the covalently cross-linked oligonucleotide sequence complementary to the universal primer site (UP-AP, Bio-Rad). The blots are hybridized for 20-30 minutes at 45°C and post hybridization washes are incubated at 45°C as two 10 minute washes in 6 M urea, 1X standard saline citrate (SSC), 0.1% SDS and one 10 minute wash in 1XSSC, 0.1% Triton™X-100. The blots are rinsed for 10 minutes at RT with 1XSSC.

Blots are incubated for 10 minutes at RT with shaking in the substrate buffer consisting of 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide, pH 10.0. Individual blots are placed in heat sealable bags with substrate buffer and 0.2 mM AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt, Bio-Rad). After a 20 minute incubation at RT with shaking, the excess AMPPD solution is removed. The blot is exposed to X-ray film overnight. Positive bands indicate the presence of the VIAF gene. Patient samples which show no

hybridizing bands lack the VIAF gene, indicating the possibility of ongoing disease such as cancer, or an enhanced susceptibility to developing a disease, such as cancer, in the future.

EXAMPLE 21

Peptide Modifications

Also disclosed are biologically active molecules that mimic the action (mimetics) of the VIAF proteins, fragments, variants and mutants disclosed herein. Synthetic embodiments of naturally-occurring peptides described herein, as well as analogues (non-peptide organic molecules), derivatives (chemically functionalized peptide molecules obtained starting with the disclosed peptide sequences) and variants (homologs) of these peptides that specifically induce apoptosis. Each peptide ligand disclosed herein is comprised of a sequence of amino acids, which may be either L- and/or D- amino acids, naturally occurring and otherwise.

Peptides may be modified by a variety of chemical techniques to produce derivatives having essentially the same activity as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the peptide, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C1-C16 ester, or converted to an amide of formula NR1R2 wherein R1 and R2 are each independently H or C1-C16 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6- membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C1-C16 alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the peptide side chain may be converted to C1-C16 alkoxy or to a C1-C16 ester using well-recognized techniques. Phenyl and phenolic rings of the peptide side chain may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with C1-C16 alkyl, C1-C16 alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide sidechains can be extended to homologous C2-C4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides disclosed herein to select and provide conformational constraints to the structure that result in enhanced stability. For example, a carboxyl-terminal or amino-terminal cysteine residue can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, thereby generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

To maintain an optimally functional peptide, particular peptide variants will differ by only a small number of amino acids from the peptides disclosed in this specification. Such variants may have deletions (for example of 1-3 or more amino acid residues), insertions (for example of 1-3 or

more residues), or substitutions that do not interfere with the desired activity of the peptides. Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. For example, such variants can have amino acid substitutions of single residues, for example 1, 3, 5 or even 10 substitutions in the full length VIAF protein (SEQ ID NOS 2, 4, 6, 8 and 10).

Peptidomimetic and organomimetic embodiments are also disclosed herein, whereby the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid side chains in the peptide, resulting in such peptido- and organomimetics of the peptides having substantial specific hair growth promoting and blocking activity. For computer modeling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press: Buffalo Grove, IL, pp. 165-174 and Principles of Pharmacology (ed. Munson, 1995), chapter 102 for a description of techniques used in CADD. Also disclosed are mimetics prepared using such techniques that produce either peptides or conventional organic pharmaceuticals that retain the biological activity of VIAF.

The above described mimetics are examined for their ability to modulate the anti-apoptotic and signaling properties of the IAP family. Such activities can be readily determined using the assays disclosed herein, for example using the methods described in EXAMPLES 7-10. Suitable mimetics would demonstrate VIAF biological activity as defined above.

EXAMPLE 22

Method for Generating Mimetics

Compounds or other molecules which mimic normal VIAF function, such as compounds which modulate the anti-apoptotic and signaling properties of the IAP family, can be identified and/or designed. These compounds or molecules are known as mimetics, because they mimic the biological activity of the normal protein.

Crystallography

To identify the amino acids that interact between the IAPs and VIAF, VIAF is co-crystallized in the presence of an IAP protein, for example, XIAP, c-IAP1 or c-IAP2. One method that can be used is the hanging drop method. In this method, a concentrated salt, IAP and VIAF protein solution is applied to the underside of a lid of a multiwell dish. A range of concentrations may need to be tested. The lid is placed onto the dish, such that the droplet "hangs" from the lid. As the solvent evaporates, a protein crystal is formed, which can be visualized with a microscope.

This crystallized structure is then subjected to X-ray diffraction or NMR analysis which allows for the identification of the amino acid residues that are in contact with one another. The amino acids that contact the transcription factors establish a pharmacophore that can then be used to identify drugs that interact at that same site.

Identification of drugs

Once these amino acids have been identified, one can screen synthetic drug databases (which can be licensed from several different drug companies), to identify drugs that interact with the same amino acids of VIAF that IAPs, such as XIAP, c-IAP1 or c-IAP2, interact with. Moreover, structure activity relationships and computer assisted drug design can be performed as described in Remington, *The Science and Practice of Pharmacy*, Chapter 28.

Designing synthetic peptides

In addition, synthetic peptides can be designed from the sequence of an IAP (such as XIAP, cIAP-1 or cIAP-2) that interacts with VIAF. Several different peptides could be generated from this region. This could be done with or without the crystallography data. However, once crystallography data is available, peptides can also be designed that bind better than VIAF.

The chimeric peptides may be expressed recombinantly, for example in *E. coli*. One advantage of the synthetic peptides over the monoclonal antibodies is that they are smaller, and therefore diffuse easier, and are not as likely to be immunogenic. Standard mutagenesis of such peptides can also be performed to identify variant peptides having even greater enhancement of transcription and splicing.

After synthetic drugs or peptides that bind to IAPs have been identified, their ability to modulate the anti-apoptotic and signaling properties of the IAP family, can be tested as described in the above EXAMPLES 7-10. Those that are positive would be good candidates for therapies, such as treatment of diseases including, but not limited to: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; 6-12 leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes, or diseases in which VIAF is underexpressed VIAF or where greater expression of VIAF is desired.

EXAMPLE 23

Peptide Synthesis and Purification

The disclosed peptides (and variants, and fragments, and mutants thereof) can be chemically synthesized by any of a number of manual or automated methods of synthesis known in the art. For example, solid phase peptide synthesis (SPPS) is carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-

fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with dicyclohexylcarbodiimide/ hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/ hydroxybenzotriazole (HBTU/HOBT), and using p-hydroxymethylphenoxymethylpolystyrene (HMP) or Sasrin resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides.

Fmoc-derivatized amino acids are prepared from the appropriate precursor amino acids by tritylation and triphenylmethanol in trifluoroacetic acid, followed by Fmoc derivitization as described by Atherton *et al.* (*Solid Phase Peptide Synthesis*, IRL Press: Oxford, 1989).

Sasrin resin-bound peptides are cleaved using a solution of 1% TFA in dichloromethane to yield the protected peptide. Where appropriate, protected peptide precursors are cyclized between the amino- and carboxyl-termini by reaction of the amino-terminal free amine and carboxyl-terminal free acid using diphenylphosphorylazide in nascent peptides wherein the amino acid sidechains are protected.

HMP or Rink amide resin-bound products are routinely cleaved and protected sidechain-containing cyclized peptides deprotected using a solution comprised of trifluoroacetic acid (TFA), optionally also comprising water, thioanisole, and ethanedithiol, in ratios of 100 : 5 : 5 : 2.5, for 0.5 - 3 hours at RT.

Crude peptides are purified by preparative high pressure liquid chromatography (HPLC), for example using a Waters Delta-Pak C18 column and gradient elution with 0.1% TFA in water modified with acetonitrile. After column elution, acetonitrile is evaporated from the eluted fractions, which are then lyophilized. The identity of each product so produced and purified may be confirmed by fast atom bombardment mass spectroscopy (FABMS) or electrospray mass spectroscopy (ESMS).

EXAMPLE 24

Pharmaceutical Compositions and Modes of Administration

Various delivery systems for administering the combined therapy disclosed herein are known, and include e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see Wu and Wu, *J. Biol. Chem.* 1987, 262:4429-32), and construction of a therapeutic nucleic acid as part of a retroviral or other vector. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with ther biologically active agents. Administration can be systemic or local. In addition, the pharmaceutical compositions may be introduced into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular

injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

In one embodiment, it may be desirable to administer the pharmaceutical compositions disclosed herein locally to the area in need of treatment, for example, by local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, through a catheter, by a suppository or an implant, such as a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

The use of liposomes as a delivery vehicle is one delivery method of interest. The liposomes fuse with the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the target cells for a sufficient time for fusion to occur, using various means to maintain contact, such as isolation and binding agents. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus. The lipids may be any useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine. Other potential lipids include neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like. For preparing the liposomes, the procedure described by Kato *et al.* (*J. Biol. Chem.* 1991, 266:3361) may be used.

The present disclosure also provides pharmaceutical compositions which include a therapeutically effective amount of the VIAF protein, RNA, DNA, antisense molecule or antibody, alone or with a pharmaceutically acceptable carrier.

Delivery systems

Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile, and the formulation suits the mode of administration. The composition can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate.

The amount of the inducing agent and disrupting agent that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease

or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The disclosure also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. Instructions for use of the composition can also be included.

The pharmaceutical compositions or methods of treatment may be administered in combination with other therapeutic treatments, such as other antineoplastic or antitumorigenic therapies.

Administration of Nucleic Acid Molecules

In an embodiment in which a VIAF nucleic acid is employed for gene therapy, the analog is delivered intracellularly (e.g., by expression from a nucleic acid vector or by receptor-mediated mechanisms). In an embodiment where the therapeutic molecule is a nucleic acid or antisense molecule, administration can be achieved by an appropriate nucleic acid expression vector which is administered so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot *et al.*, *Proc. Natl. Acad. Sci. USA* 1991, 88:1864-8), etc. Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The vector pcDNA, is an example of a method of introducing the foreign cDNA into a cell under the control of a strong viral promoter (CMV) to drive the expression. However, other vectors can be used (see EXAMPLES 14, 18, and 19). Other retroviral vectors (such as pRETRO-ON, Clontech), also use this promoter but have the advantages of entering cells without any transfection aid, integrating into the genome of target cells ONLY when the target cell is dividing (as cancer cells do, especially during first remissions after chemotherapy) and they are regulated. It is also possible to turn on the expression of the VIAF nucleic acid by administering tetracycline when these plasmids are used. Hence these plasmids can be allowed to transfect the cells, then administer a course of tetracycline with a course of chemotherapy to achieve better cytotoxicity.

Other plasmid vectors, such as pMAM-neo (Clontech) or pMSG (Pharmacia) use the MMTV-LTR promoter (which can be regulated with steroids) or the SV10 late promoter (pSVL, Pharmacia) or metallothionein-responsive promoter (pBPV, Pharmacia) and other viral vectors,

including retroviruses. Examples of other viral vectors include adenovirus, AAV (adeno-associated virus), recombinant HSV, poxviruses (vaccinia) and recombinant lentivirus (such as HIV). These vectors achieve the basic goal of delivering into the target cell the cDNA sequence and control elements needed for transcription. The present disclosure includes all forms of nucleic acid delivery, including synthetic oligos, naked DNA, plasmid and viral, integrated into the genome or not.

Administration of Antibodies

In an embodiment where the therapeutic molecule is an antibody, specifically an antibody that recognizes VIAF protein, administration may be achieved by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents. Similar methods can be used to administer VIAF protein, of fragments thereof.

The disclosure also provides pharmaceutical compositions which include a therapeutically effective amount of the antibody, and a pharmaceutically acceptable carrier or excipient.

EXAMPLE 25

Disruption of VIAF Expression

This example describes methods that can be used to disrupt VIAF expression. Such methods are useful when apoptosis is desired, for example in the pathogenesis of proliferative disorders, for example neoplasms, such as cancer. In addition, in *in vitro* systems, it has been shown that apoptosis is used to fight viral infections. Furthermore, apoptosis is necessary for normal embryonal development. One approach to disrupting VIAF function or expression is to use antisense oligonucleotides.

To design an antisense oligonucleotide, the mRNA sequence from the desired molecule, such as human VIAF, is examined. Regions of the sequence containing multiple repeats, such as TTTTTTTT, are not as desirable because they will lack specificity. Several different regions can be chosen. Of those, oligos are selected by the following characteristics: ones having the best conformation in solution; ones optimized for hybridization characteristics; and one having less potential to form secondary structures. Antisense molecules having a propensity to generate secondary structures are less desirable.

Plasmids containing VIAF antisense sequences can also be genereated. For example, cDNA fragments coding for human VIAF are PCR amplified. The nucleotides are then amplified using Pfu DNA polymerase (Stratagene) and cloned in antisense orientation a vector, such as pcDNA vectors (InVitrogen, Carlsbad CA). The nucleotide sequence and orientation of the insert can be confirmed by dideoxy sequencing using a Sequenase kit (Amersham Pharmacia Biotech).

Generally, the term "antisense" refers to a nucleic acid capable of hybridizing to a portion of a VIAF RNA (such as mRNA) by virtue of some sequence complementarity. The antisense nucleic acids disclosed herein can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

The VIAF antisense nucleic acids are polynucleotides, and may be oligonucleotides (ranging from 6 to about 100 oligonucleotides). In specific aspects, the oligonucleotide is at least 10, 15, or 100 nucleotides, or a polynucleotide of at least 200 nucleotides. The antisense nucleic acids may be much longer constructs. The nucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The nucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, and may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, e.g., Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, 86:6553-6; Lemaitre *et al.*, *Proc. Natl. Acad. Sci. USA* 1987, 84:648-52; PCT Publication No. WO 88/09810) or blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134), hybridization triggered cleavage agents (see, e.g., Krol *et al.*, *BioTechniques* 1988, 6:958-76) or intercalating agents (see, e.g., Zon, *Pharm. Res.* 1988, 5:539-49).

In one embodiment disclosed herein, a VIAF antisense polynucleotide (including oligonucleotides) is provided, for example of single-stranded DNA. The VIAF antisense polynucleotide may recognize any species of VIAF. The antisense polynucleotide may be modified at any position on its structure with substituents generally known in the art. For example, a modified base moiety may be 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N~6-sopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, methoxyarninomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-S-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

In another embodiment, the polynucleotide includes at least one modified sugar moiety such as arabinose, 2-fluoroarabinose, xylose, and hexose, or a modified component of the phosphate backbone, such as phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, or a formacetal or analog thereof.

In yet another embodiment, the polynucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.* 1987, 15:6625-41). The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent. Oligonucleotides may include a targeting moiety that enhances uptake of the molecule by tumor cells. The targeting moiety may be a specific binding molecule, such as an antibody or fragment thereof that recognizes a molecule present on the surface of the tumor cell.

As an alternative to antisense inhibitors, catalytic nucleic acid compounds, such as ribozymes or anti-sense conjugates, may be used to inhibit gene expression. Ribozymes may be synthesized and administered to the subject, or may be encoded on an expression vector, from which the ribozyme is synthesized in the targeted cell (as in PCT publication WO 9523225, and Beigelman *et al. Nucl. Acids Res.* 1995, 23:4434-42). Examples of oligonucleotides with catalytic activity are described in WO 9506764. Conjugates of antisense with a metal complex, e.g. terpyridylCu (II), capable of mediating mRNA hydrolysis, are described in Bashkin *et al.*, *Appl. Biochem Biotechnol.* 1995, 54:43-56.

Polynucleotides disclosed herein can be synthesized by standard methods known in the art, for example by use of an automated DNA synthesizer (Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligos may be synthesized by the method of Stein et al. (*Nucl. Acids Res.* 1998, 16:3209), methylphosphonate oligos can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:7448-51). In a specific embodiment, the VIAF antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see PCT International Publication WO 90/11364, Sarver et al., *Science* 1990, 247:1222-5). In another embodiment, the oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., *Nucl. Acids Res.* 1987, 15:6131-48), or a chimeric RNA-DNA analogue (Inoue et al., *FEBS Lett.* 1987, 215:327-330).

The antisense polynucleic acids disclosed herein comprise a sequence complementary to at least a portion of an RNA transcript of a *VIAF* gene, such as a human *VIAF* gene. However, absolute complementarity, although advantageous, is not required. A sequence may be complementary to at least a portion of an RNA, meaning a sequence having sufficient complementarily to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded VIAF antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a VIAF RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable

degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The relative ability of polynucleotides (such as oligonucleotides) to bind to complementary strands is compared by determining the melting temperature (T_m) of a hybridization complex of the poly/oligonucleotide and its complementary strand. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). A reduction in UV absorption indicates a higher T_m. The higher the T_m the greater the strength of the binding of the hybridized strands. As close to optimal fidelity of base pairing as possible achieves optimal hybridization of a poly/oligonucleotide to its target RNA.

The amount of VIAF antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In one embodiment, pharmaceutical compositions comprising VIAF antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In other embodiments, it may be useful to use such compositions to achieve sustained release of the VIAF antisense nucleic acids. In yet another embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti *et al. Proc. Natl. Acad. Sci. USA* 1990, 87:2448-51; Renneisen *et al. J. Biol. Chem.* 1990, 265:16337-42).

EXAMPLE 26

Methods of Treatment using Antisense Molecules

When VIAF levels are prematurely downregulated by various antisense strategies, the cells maybe induced into entering an apoptotic pathway. VIAF antisense oligonucleotides (EXAMPLE 25) can be used to disrupt cellular expression of a VIAF protein.

The subject suffering from a disease in which apoptosis is desired, can be treated with a therapeutically effective amount of VIAF antisense. After the VIAF antisense has taken affect (VIAF levels are downregulated), after 24-48 hours, the subject can be monitored for decreased apoptosis.

Prophylactic Treatments

The treatments disclosed herein can also be used prophylactially, for example to inhibit or prevent progression to of a disease in which apoptosis is is not desired. Such administration is indicated where the treatment is shown to have utility for treatment or prevention of the disorder. The prophylactic use is indicated in conditions known or suspected of preceding progression to diseases associated with an undesired amount of apoptosis, for example in diseases associated with VIAF expression. Such diseases may include cancer, heterocellular hereditary persistence of fetal

hemoglobin, deafness, cardiomyopathy, 6-12 leukemia, MALT lymphoma, and insulin dependent diabetes.

EXAMPLE 27

Cloning of VIAF Genomic DNA

Methods for cloning VIAF genomic DNA from any species are known to those skilled in the art, and are described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. 1989. Herein incorporated by reference). Briefly, VIAF cDNA (full length or fragments thereof, for example SEQ ID NOs 1, 3, 5, 7, and 9) is radiolabeled as described in EXAMPLE 4 with Rediprime II (Amersham Pharmacia Biotech) as instructed by the manufacturer. The radiolabeled cDNA is used to screen a bacteriophage lamda gt11 genomic library. Genomic DNA of the resulting positive clones is isolated, purified and digested with appropriate restriction enzymes. Digested DNA is separated by agarose gel electrophoresis and blotted onto a nylon membrane. A Southern-Blot is performed using radioactive cDNA of VIAF to identify the exons. Bands that hybridized with the cDNA are isolated from the gel and sequenced. The resulting DNA sequence is analyzed by specific computer programs to identify the promoter region and exon/intron donor/acceptor sites.

EXAMPLE 28

VIAF Transgenic Plants and Animals

The creation of transgenic plants and animals which express VIAF can be made by techniques known in the art, for example those disclosed in U.S. Patent Nos. 5,574,206; 5,723,719; 5,175,383; 5,824,838; 5,811,633; 5,620,881; and 5,767,337, which are incorporated by reference.

Methods for generating transgenic mice are described in *Gene Targeting*, Joyuner ed., Oxford University Press, 1995 and Watson *et al.*, *Recombinant DNA 2nd Ed.*, W.H. Freeman and Co., New York, 1992, Chapter 14. To generate transgenic mice containing a functional deletion of the VIAF gene, genomic fragments can be used as short arm and long arm. Between long arm and short arm, the *neo* gene is introduced, generating a the knock-out vector.

Using standard transgenic mouse technology, the knock-out vector can be used to generate VIAF knock-out mice by homologous recombination. The knock-out vector is introduced into embryonic stem cells (ES cells) by standard methods which may include transfection, retroviral infection or electroporation (also see EXAMPLE 14). Transfected ES cells expressing the knock-out vector will grow in medium containing the antibiotic G418. The neomycin resistant ES cells are microinjected into mouse embryos (blastocysts), which are implanted into the uterus of pseudopregnant mice. The resulting litter is screened for chimeric mice by observing their coat color. Chimeric mice are ones in which the injected ES cells developed into the germ line, thereby

allowing transmission of the gene to their offspring. The resulting heterozygotic mice will be mated to generate a homozygous line of transgenic mice functionally deleted for VIAF. These homozygous mice will then be screened phenotypically, for example, their predisposition to developing diseases which can include: cancer, heterocellular hereditary persistence of fetal hemoglobin, deafness, cardiomyopathy, 6-12 leukemia, MALT lymphoma, diabetes mellitus, and insulin dependent diabetes. Knock-out mice which do not express VIAF in their cells can be prepared to further investigate the role of VIAF on apoptosis pathways.

EXAMPLE 30

Generation and Expression of VIAF Fusion Proteins

Methods for making fusion proteins are well known to those skilled in the art. For example U.S. Patent No. 6,057,133 to Bauer *et al.* (herein incorporated by reference) discloses methods for making fusion molecules composed of human interleukin-3 (hIL-3) variant or mutant proteins functionally joined to a second colony stimulating factor, cytokine, lymphokine, interleukin, hematopoietic growth factor or IL-3 variant. U.S. Patent No. 6,072,041 to Davis *et al.* (herein incorporated by reference) discloses the generation of fusion proteins comprising a single chain Fv molecule directed against a transcytotic receptor covalently linked to a therapeutic protein.

Similar methods can be used to generate fusion proteins comprising VIAF (or variants or fragments thereof) linked to other amino acid sequences. Linker regions can be used to space the two portions of the protein from each other and to provide flexibility between them. The linker region is generally a polypeptide of between 1 and 500 amino acids in length, for example less than 30 amino acid in length. The linker joining the two molecules can be designed to (1) allow the two molecules to fold and act independently of each other, (2) not have a propensity for developing an ordered secondary structure which could interfere with the functional domains of the two proteins, (3) have minimal hydrophobic or charged characteristic which could interact with the functional protein domains and (4) provide steric separation of the two regions. Typically surface amino acids in flexible protein regions include Gly, Asn and Ser. Other neutral amino acids, such as Thr and Ala, can also be used in the linker sequence. Additional amino acids may also be included in the linker due to the addition of unique restriction sites in the linker sequence to facilitate construction of the fusions. Other moieties may also be included, as desired. These may include a binding region, such as avidin or an epitope, such as a polyhistadine tag, which may be useful for purification and processing of the fusion protein. In addition, detectable markers can be attached to the fusion protein, so that the traffic of the fusion protein through a body or cell may be monitored conveniently. Such markers may include radionuclides, enzymes, fluors, and the like.

Fusing of the nucleic acid sequences of VIAF (or variant or fragment thereof), with the nucleic acid sequence of another protein (or variant or fragment thereof), can be accomplished by

the use of intermediate vectors. Alternatively, one gene can be cloned directly into a vector containing the other gene. Linkers and adapters can be used for joining the nucleic acid sequences, as well as replacing lost sequences, where a restriction site was internal to the region of interest. Genetic material (DNA) encoding one polypeptide, peptide linker, and the other polypeptide is inserted into a suitable expression vector which is used to transform prokaryotic or eukaryotic cells, for example bacteria, yeast, insect cells or mammalian cells (see EXAMPLE 14). The transformed organism is grown and the protein isolated by standard techniques, for example by using a detectable marker such as nickel-chelate affinity chromatography, if a polyhistadine tag is used. The resulting product is therefore a new protein, a fusion protein, which has a VIAF joined by a linker region to a second protein. To confirm that the fusion protein was expressed, the purified protein is subjected to electrophoresis in SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane filters using established methods. The protein products can be identified by Western blot analysis using antibodies directed against the individual components, i.e., polyhistadine tag and VIAF (see EXAMPLES 5 and 15).

Having illustrated and described the principles of isolating several VIAF cDNAs, the proteins they encode, antibodies which recognize the proteins and modes of use of these biological molecules to modulate the anti-apoptotic and signaling properties of IAP family members, such as XIAP, it should be apparent to one skilled in the art that the disclosure can be modified in arrangement and detail without departing from such principles. In view of the many possible embodiments to which the principles of our disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. Rather, the scope of the disclosure is in accord with the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A purified or synthetic protein having VIAF biological activity, and comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 2, 4, 6 or 8;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

2. The protein of claim 1, wherein the amino acid sequence comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 2;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence s of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

3. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 4;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

4. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 6;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

5. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 8;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

6. The protein of claim 1(d), wherein the amino acid sequences have at least 90% sequence identity to the sequence specified in 1(a).

7. The protein of claim 1(d), wherein the amino acid sequences have at least 95% sequence identity to the sequence specified in 1(a).

8. The protein of claim 1(d), wherein the amino acid sequences have at least 98% sequence identity to the sequence specified in 1(a).

9. The protein of claim 1, wherein the amino acid sequence contains no more than 270 amino acid residues.

10. The protein of claim 1, wherein the amino acid sequence contains no more than 260 amino acid residues.

11. The protein of claim 1, wherein the amino acid sequence contains no more than 250 amino acid residues.

12. An animal protein having VIAF biological activity.

13. The animal protein of claim 12, wherein the animal is a mammal.

14. The animal protein of claim 12, wherein the mammal is a human.

15. An isolated nucleic acid molecule encoding a protein according to any of claims 1-10.

16. The isolated nucleic acid of claim 15, further comprising a promoter sequence operably linked to the nucleic acid of claim 15.

17. An isolated nucleic acid molecule, wherein the nucleic acid molecule includes the sequence selected from the group consisting of:

(a) SEQ ID NO 1 or its complementary strand;

(b) SEQ ID NO 3 or its complementary strand;

(c) SEQ ID NO 5 or its complementary strand;

(d) SEQ ID NO 7 or its complementary strand; and

(e) Sequences which hybridize under conditions of at least 75% stringency to the sequences defined in (a), (b), (c), or (d).

18. The isolated nucleic acid molecule of claim 17, wherein the nucleic acid comprises the nucleic acid sequence depicted as nucleotides 62-781 of SEQ ID NO 1.

19. The isolated nucleic acid molecule of claim 17, wherein the nucleic acid includes a sequence selected from the group consisting of:

(a) at least 370 contiguous nucleotides of SEQ ID NO 1 or its complementary strand;

(b) at least 20 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(c) at least 30 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(d) at least 50 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(e) at least 21 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(f) at least 30 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(g) at least 50 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(h) at least 22 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(i) at least 30 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(j) at least 50 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(k) at least 21 contiguous nucleotides of SEQ ID NO 7 or its complementary strand;

(l) at least 30 contiguous nucleotides of SEQ ID NO 7 or its complementary strand; and

(m) at least 50 contiguous nucleotides of SEQ ID NO 7 or its complementary strand.

20. An isolated nucleic acid molecule that:

(a) is at least 80% homologous to SEQ ID NOS 1, 3, 5, or 7; and

(b) encodes a protein having VIAF biological activity.

21. A recombinant vector including a nucleic acid molecule according to any of claims 15-20.

22. A transgenic cell produced by introducing into a cell a recombinant vector according to claim 21.

23. A purified protein encoded by the nucleic acid molecule according to any of claims 15-20.

24. The protein of claim 23, wherein the protein has an amino acid sequence as shown in SEQ ID NO 2, 4, 6, or 8.

25. The isolated nucleic acid molecule of any of claims 15-20 wherein the nucleic acid molecule encodes a peptide having VIAF biological activity.

26. The isolated nucleic acid molecule according to claim 17(e) wherein the nucleic acid molecule hybridizes under conditions of at least 90% stringency to the sequences defined in claim 17 (a), claim 17 (b), claim 17(c), or claim 17 (d).

27. An antisense oligonucleotide which:

hybridizes to an RNA or a plus strand of a nucleic acid any of claims 15-20; and

inhibits VIAF biological activity.

28. A specific binding agent capable of specifically binding to a VIAF protein.

29. The specific binding agent of claim 28 wherein the specific binding agent is selected from the group consisting of: polyclonal antibodies; monoclonal antibodies; and fragments of monoclonal antibodies.

30. A composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of the protein of any of claims 1-14 or 23-24.

31. The composition of claim 30, further comprising one or more other anti-apoptotic compounds.

32. A composition comprising a therapeutically effective amount of the specific binding agent of claim 28, and a pharmaceutically acceptable carrier.

33. The compositions of any of claims 30-32, for use in decreasing apoptosis.

34. The composition of claim 33, for use in a subject suffering from unwanted apoptosis, in an amount sufficient to inhibit Bax- and Fas-induced apoptosis in the subject.

35. The composition of claim 34, wherein the subject has a cancer, an autoimmune disease, or a neurodegenerative disease characterized by unwanted apoptosis.

36. A composition comprising a therapeutically effective amount of the antisense oligonucleotide of claim 27, and a pharmaceutically acceptable carrier.

37. The composition of claim 36, for use in a subject with a disease, in whom apoptosis is desired.

38. A method for detecting an enhanced susceptibility of a subject to a disease of abnormal apoptosis, the method comprising detecting a deletion of or within a VIAF gene in cells of the subject, or detecting a decrease or absence of VIAF protein in cells of the subject.

39. The method of claim 38 wherein the disease is selected from the group consisting of: cancers, autoimmune diseases; and neurodegenerative diseases.

40. The method of claim 38, wherein the method comprises detecting a deletion of or within a VIAF gene in a cell, the method comprising:

incubating the nucleic acid of claim 17 with a nucleic acid of the cell under conditions such that the nucleic acid will specifically hybridize to the VIAF gene present in the nucleic acid to form an nucleic acid:VIAF gene complex;

detecting an increase or decrease of the nucleic acid:VIAF complexes, wherein an absence of the complexes indicates deletion of or within the VIAF gene.

41. The method of claim 38, wherien the method comprises:

incubating the specific binding agent of claim 28 with proteins of the cell under conditions such that the specific binding agent will specifically bind to a VIAF protein present in the cell to form a specific binding agent:VIAF protein complex; and

detecting an increase or decrease of specific binding agent:VIAF protein complexes.

42. A method of treating a disease of abnormal apoptosis by supplying VIAF biological activity to a cell which has lost the VIAF biological activity by a deletion of all or a portion of a VIAF gene, comprising introducing the nucleic acid of claim 10 into the cell such that the nucleic acid of claim 17 is expressed in the cell.

43. A method of decreasing apoptosis in a cell comprising increasing the level of VIAF biological activity, which prevents the cell from undergoing apoptosis.

44. The method of claim 43, whereby decreasing apoptosis inhibits Bax- and Fas-induced apoptosis.

45. The method of claim 44, wherein the cell is characterized by:

(a) decreased expression of a VIAF protein; or

(b) decreased VIAF biological activity or expression, relative to VIAF biological activity or expression in a same tissue type that is undergoing apoptosis.

46. The method of claim 45, wherein increasing the level of VIAF biological activity comprises exposing the cell to a therapeutically effective amount of the VIAF protein of claim 1.

47. The method of claim 46, wherein increasing the level of VIAF biological activity comprises administering a therapeutically effective amount of a nucleic acid which can express a protein having VIAF biological activity.

48. The method of claim 47, wherein the nucleic acid is the nucleic acid of claim 13.

49. The method of claim 43 or the composition of claim 33, wherein decreasing apoptosis treats a disease caused by defects in apoptosis.

50. The method of claim 49 or the composition of claim 34 wherein the disease is selected from the group consisting of autoimmune and neurodegenerative diseases

51. The method and composition of claim 50, wherein the disease is selected from the group consisting of: diabetes, multiple sclerosis and retinal degeneration.

52. A method for inducing apoptosis by adding a therapeutically effective amount of the antisense oligonucleotide of claim 27 sufficient to induce apoptosis in a subject.

53. The method of claim 52 and the composition of claim 37, wherein apoptosis is induced to treat a disease caused by defects in apoptosis.

54. The method and composition of claim 53, wherein the disease is a cancer.

55. A method of treating a disease caused by a mutation in the nucleic acid of claim 17 by supplying therapeutically effective amounts of a polypeptide product or the nucleic acid.

56. The protein of claim 1, for use in modulating apoptosis.

57. The nucleic acid of claim 15, for use in modulating apoptosis.

58. The protein of claim 1, for use in inhibiting apoptosis.

59. The antisense oligonucleotide of claim 27, for use in increasing apoptosis.

FIG. 1

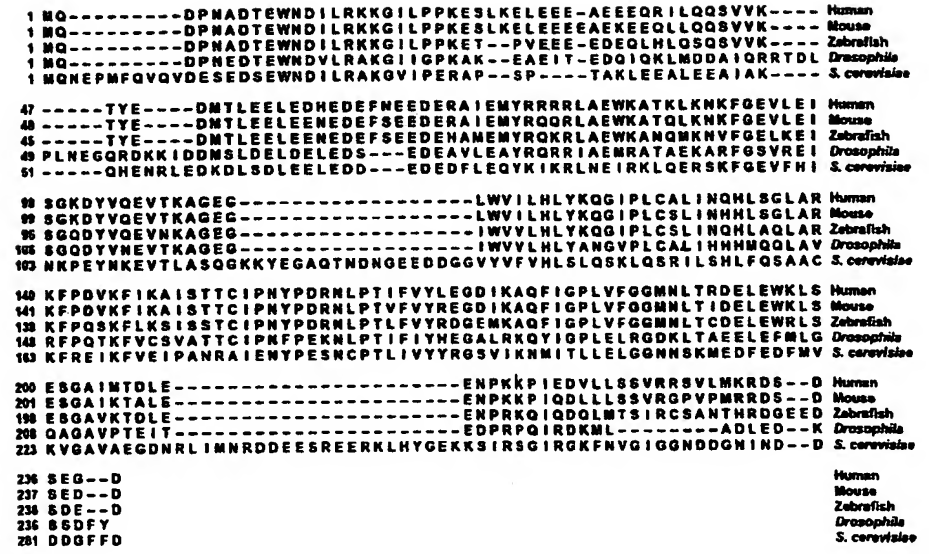


FIG. 3

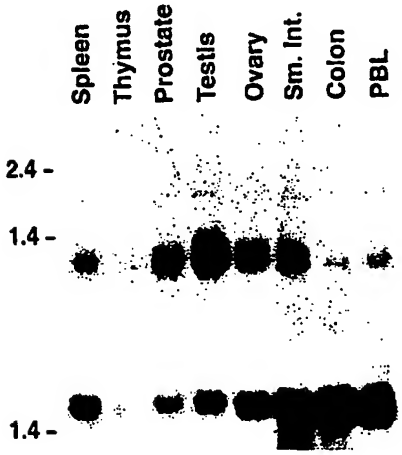


FIG. 4

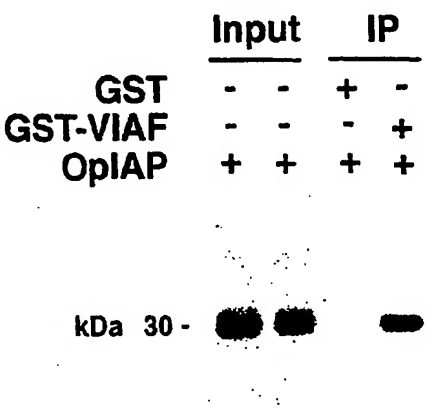


FIG. 2

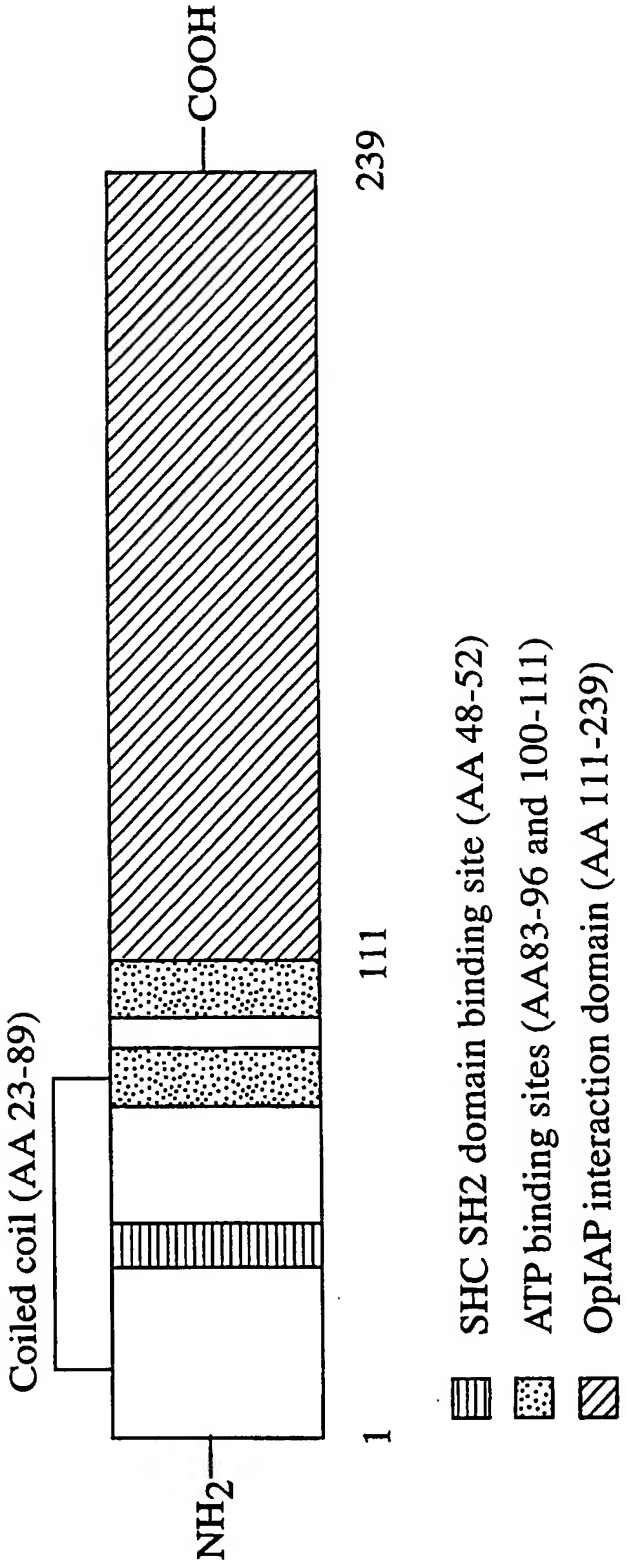

COOH
239
111
1
NH_2
Coiled coil (AA 23-89)
SHC SH2 domain binding site (AA 48-52)
ATP binding sites (AA83-96 and 100-111)
OpIAP interaction domain (AA 111-239)

FIG. 5

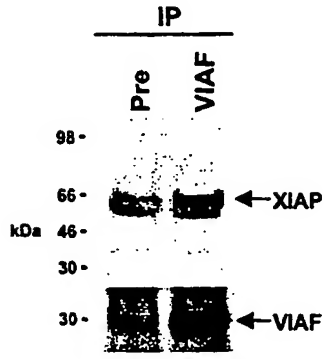


FIG. 6

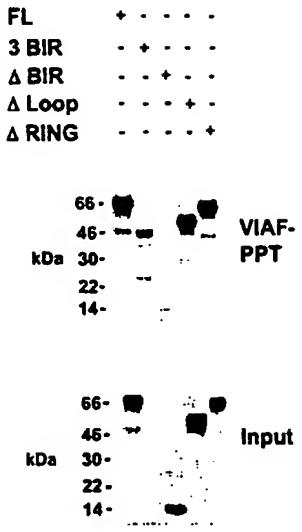


FIG. 7

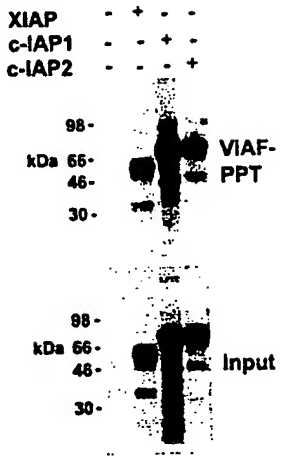


FIG. 8A

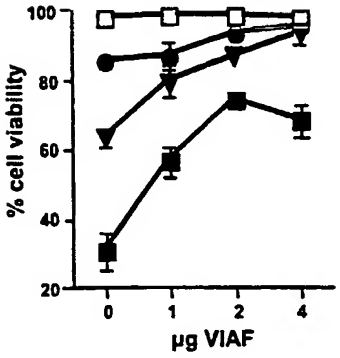


FIG. 8B

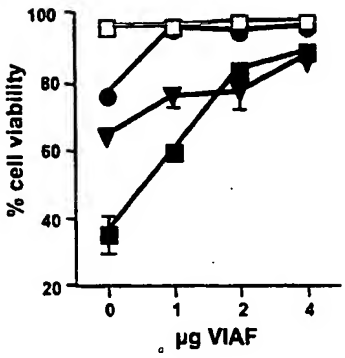


FIG. 9

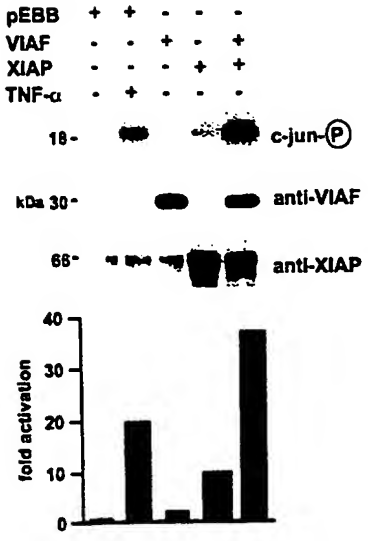
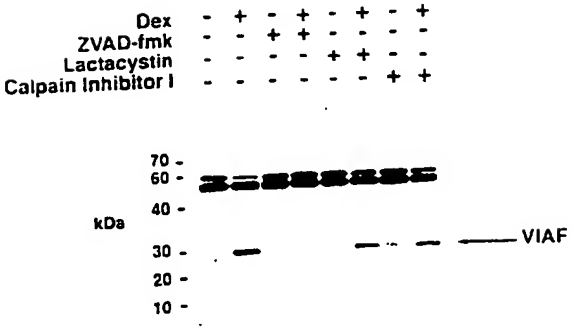


FIG. 10



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<120> Cloning and Characterization of VIAF in Several Organisms

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<211> 1016
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (62)..(781)

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g atg cag gac ccc aac gca gac act gaa tgg aat gac atc tta cgc aaa 109
Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
1 5 10 15

aag ggt atc tta ccc ccc aag gaa agt ctg aaa gaa ttg gaa gag gag 157
Lys Gly Ile Leu Pro Pro Lys Glu Ser Leu Lys Glu Leu Glu Glu Glu
20 25 30

gca gaa gag gag cag cgc atc ctc cag cag tca gtg gtg aaa aca tat 205
Ala Glu Glu Glu Gln Arg Ile Leu Gln Gln Ser Val Val Lys Thr Tyr
35 40 45

gaa gat atg act ttg gaa gag ctg gag gat cat gaa gac gag ttt aat 253
Glu Asp Met Thr Leu Glu Glu Leu Glu Asp His Glu Asp Glu Phe Asn
50 55 60

gag gag gat gaa cgt gct att gaa atg tac aga cgg cgg aga ctg gct 301
Glu Glu Asp Glu Arg Ala Ile Glu Met Tyr Arg Arg Arg Arg Leu Ala
65 70 75 80

gag tgg aaa gca act aaa ctg aag aat aaa ttt gga gaa gtt ttg gag 349
Glu Trp Lys Ala Thr Lys Leu Lys Asn Lys Phe Gly Glu Val Leu Glu
85 90 95

atc tca ggg aag gat tat gtt caa gaa gtt acc aaa gct ggc gag ggc 397
Ile Ser Gly Lys Asp Tyr Val Gln Glu Val Thr Lys Ala Gly Glu Gly
100 105 110

ttg tgg gtc atc ttg cac ctt tac aaa caa gga att ccc ctc tgt gcc 445
Leu Trp Val Ile Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys Ala

```
        115                 120                 125

ctg ata aat cag cac ctc agt gga ctt gcc agg aag ttt cct gat gtc   493
Leu Ile Asn Gln His Leu Ser Gly Leu Ala Arg Lys Phe Pro Asp Val
    130                 135                 140

aaa ttt atc aaa gcc att tca aca acc tgc ata ccc aat tat cct gat   541
Lys Phe Ile Lys Ala Ile Ser Thr Thr Cys Ile Pro Asn Tyr Pro Asp
145                 150                 155                 160

agg aat ctg ccc acg ata ttt gtt tac ctg gaa gga gat atc aag gct   589
Arg Asn Leu Pro Thr Ile Phe Val Tyr Leu Glu Gly Asp Ile Lys Ala
                165                 170                 175

cag ttt att ggt cct ctg gtg ttt ggc ggc atg aac ctg aca aga gat   637
Gln Phe Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Arg Asp
            180                 185                 190

gag ttg gaa tgg aaa ctg tct gaa tct gga gca att atg aca gac ctg   685
Glu Leu Glu Trp Lys Leu Ser Glu Ser Gly Ala Ile Met Thr Asp Leu
        195                 200                 205

gag gaa aac cct aag aag ccg att gaa gac gtg ttg ctg tcc tca gtg   733
Glu Glu Asn Pro Lys Lys Pro Ile Glu Asp Val Leu Leu Ser Ser Val
    210                 215                 220

cgg cgc tct gtc ctc atg aag agg gac agc gat tcc gag ggt gac tga   781
Arg Arg Ser Val Leu Met Lys Arg Asp Ser Asp Ser Glu Gly Asp
225                 230                 235                 240

ggctacagct tctatcacat gccgaacttt cttgtgacaa attgtctgga ttttttaaaa 841

aaggaaaaag caagaatgaa tccttgtggt ttttagtttt gtataaatta tgtttcaaat 901

ctttacattt tggaaataat cattgctgga gattctgtta aatattttgg aactcttttt 961

tttttaaatt atagtatttc ctctaaaaaa aattaaaacc agccatttgt atggc      1016
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<210> 2
<211> 239
<212> PRT
<213> Homo sapiens

<400> 2

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Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
  1               5                  10                  15
Lys Gly Ile Leu Pro Pro Lys Glu Ser Leu Lys Glu Leu Glu Glu Glu
             20                  25                  30
Ala Glu Glu Glu Gln Arg Ile Leu Gln Gln Ser Val Val Lys Thr Tyr
         35                  40                  45
Glu Asp Met Thr Leu Glu Glu Leu Glu Asp His Glu Asp Glu Phe Asn
     50                  55                  60
Glu Glu Asp Glu Arg Ala Ile Glu Met Tyr Arg Arg Arg Arg Leu Ala
 65                  70                  75                  80
Glu Trp Lys Ala Thr Lys Leu Lys Asn Lys Phe Gly Glu Val Leu Glu
                 85                  90                  95
Ile Ser Gly Lys Asp Tyr Val Gln Glu Val Thr Lys Ala Gly Glu Gly
            100                 105                 110
Leu Trp Val Ile Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys Ala
        115                 120                 125
```

```
Leu Ile Asn Gln His Leu Ser Gly Leu Ala Arg Lys Phe Pro Asp Val
    130                 135                 140
Lys Phe Ile Lys Ala Ile Ser Thr Thr Cys Ile Pro Asn Tyr Pro Asp
145                 150                 155                 160
Arg Asn Leu Pro Thr Ile Phe Val Tyr Leu Glu Gly Asp Ile Lys Ala
                165                 170                 175
Gln Phe Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Arg Asp
            180                 185                 190
Glu Leu Glu Trp Lys Leu Ser Glu Ser Gly Ala Ile Met Thr Asp Leu
        195                 200                 205
Glu Glu Asn Pro Lys Lys Pro Ile Glu Asp Val Leu Leu Ser Ser Val
    210                 215                 220
Arg Arg Ser Val Leu Met Lys Arg Asp Ser Asp Ser Glu Gly Asp
225                 230                 235
```

<210> 3
<211> 723
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(723)

<400> 3

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atg cag gac ccc aat gca gac acc gag tgg aat gac atc cta cgt aaa   48
Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
  1               5                  10                  15

aag ggc atc ctt ccc ccg aag gag agc ctg aag gag ctg gag gag gag   96
Lys Gly Ile Leu Pro Pro Lys Glu Ser Leu Lys Glu Leu Glu Glu Glu
            20                  25                  30

gag gcg gag aag gag gag cag ctc ctc cag cag tca gtg gtg aaa aca   144
Glu Ala Glu Lys Glu Glu Gln Leu Leu Gln Gln Ser Val Val Lys Thr
        35                  40                  45

tac gag gac atg act ctg gaa gag ctg gag gag aac gag gat gag ttc   192
Tyr Glu Asp Met Thr Leu Glu Glu Leu Glu Glu Asn Glu Asp Glu Phe
    50                  55                  60

agt gag gag gat gaa cga gct atc gag atg tac cgg caa cag agg ttg   240
Ser Glu Glu Asp Glu Arg Ala Ile Glu Met Tyr Arg Gln Gln Arg Leu
65                  70                  75                  80

gct gag tgg aaa gca act cag ctg aag aac aaa ttt gga gaa gtt tta   288
Ala Glu Trp Lys Ala Thr Gln Leu Lys Asn Lys Phe Gly Glu Val Leu
                85                  90                  95

gag atc tca gga aag gac tat gtt caa gaa gtt acg aaa gcc ggc gag   336
Glu Ile Ser Gly Lys Asp Tyr Val Gln Glu Val Thr Lys Ala Gly Glu
            100                 105                 110

ggc ctg tgg gtg atc tta cac ctg tac aaa caa ggg att ccc ctc tgt   384
Gly Leu Trp Val Ile Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys
        115                 120                 125

tcc ttg ata aac cat cac ttg agt gga ctc gcc agg aag ttt ccc gat   432
Ser Leu Ile Asn His His Leu Ser Gly Leu Ala Arg Lys Phe Pro Asp
```

```
    130                 135                 140

gtg aaa ttt atc aaa gcc att tca acg acc tgc ata ccc aac tac ccc   480
Val Lys Phe Ile Lys Ala Ile Ser Thr Thr Cys Ile Pro Asn Tyr Pro
145                 150                 155                 160

gac agg aat ctc ccc acg gtg ttc gtc tac cgg gaa ggg gat atc aag   528
Asp Arg Asn Leu Pro Thr Val Phe Val Tyr Arg Glu Gly Asp Ile Lys
                165                 170                 175

gca cag ttc att ggt cct ctg gtg ttc ggt ggc atg aac ctg acc ata   576
Ala Gln Phe Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Ile
            180                 185                 190

gac gag ttg gag tgg aaa ctg tct gag tca gga gcg atc aag aca gcc   624
Asp Glu Leu Glu Trp Lys Leu Ser Glu Ser Gly Ala Ile Lys Thr Ala
        195                 200                 205

ctg gag gag aac ccc aag aag ccc atc cag gac ctg ctg ctg tcc tca   672
Leu Glu Glu Asn Pro Lys Lys Pro Ile Gln Asp Leu Leu Leu Ser Ser
    210                 215                 220

gtc cgg ggc cct gtc ccc atg agg agg gac agt gat tct gag gac gac   720
Val Arg Gly Pro Val Pro Met Arg Arg Asp Ser Asp Ser Glu Asp Asp
225                 230                 235                 240

taa                                                               723
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<210> 4
<211> 240
<212> PRT
<213> Mus musculus

<400> 4

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Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
  1               5                  10                  15
Lys Gly Ile Leu Pro Pro Lys Glu Ser Leu Lys Glu Leu Glu Glu Glu
             20                  25                  30
Glu Ala Glu Lys Glu Glu Gln Leu Leu Gln Gln Ser Val Val Lys Thr
         35                  40                  45
Tyr Glu Asp Met Thr Leu Glu Glu Leu Glu Glu Asn Glu Asp Glu Phe
     50                  55                  60
Ser Glu Glu Asp Glu Arg Ala Ile Glu Met Tyr Arg Gln Gln Arg Leu
 65                  70                  75                  80
Ala Glu Trp Lys Ala Thr Gln Leu Lys Asn Lys Phe Gly Glu Val Leu
                 85                  90                  95
Glu Ile Ser Gly Lys Asp Tyr Val Gln Glu Val Thr Lys Ala Gly Glu
            100                 105                 110
Gly Leu Trp Val Ile Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys
        115                 120                 125
Ser Leu Ile Asn His His Leu Ser Gly Leu Ala Arg Lys Phe Pro Asp
    130                 135                 140
Val Lys Phe Ile Lys Ala Ile Ser Thr Thr Cys Ile Pro Asn Tyr Pro
145                 150                 155                 160
Asp Arg Asn Leu Pro Thr Val Phe Val Tyr Arg Glu Gly Asp Ile Lys
                165                 170                 175
Ala Gln Phe Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Ile
            180                 185                 190
Asp Glu Leu Glu Trp Lys Leu Ser Glu Ser Gly Ala Ile Lys Thr Ala
```

```
       195                 200                 205
Leu Glu Glu Asn Pro Lys Lys Pro Ile Gln Asp Leu Leu Leu Ser Ser
    210                 215                 220
Val Arg Gly Pro Val Pro Met Arg Arg Asp Ser Asp Ser Glu Asp Asp
225                 230                 235                 240
```

<210> 5
<211> 720
<212> DNA
<213> Danio rerio

<220>
<221> CDS
<222> (1)..(720)

<400> 5

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atg cag gac cca aac gca gac acc gag tgg aac gat atc ctg agg aag   48
Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
  1               5                  10                  15

aag ggg att ctg cct ccg aaa gaa acg ccg gtg gag gaa gaa gag gat   96
Lys Gly Ile Leu Pro Pro Lys Glu Thr Pro Val Glu Glu Glu Glu Asp
             20                  25                  30

gag cag ctt cat ctt cag tct cag tct gtg gtg aag acc tat gag gac   144
Glu Gln Leu His Leu Gln Ser Gln Ser Val Val Lys Thr Tyr Glu Asp
         35                  40                  45

atg acc ctg gag gag ctg gag gag aat gaa gac gag ttc agt gaa gaa   192
Met Thr Leu Glu Glu Leu Glu Glu Asn Glu Asp Glu Phe Ser Glu Glu
     50                  55                  60

gat gag cac gct atg gag atg tac aga cag aag agg ctg gcg gag tgg   240
Asp Glu His Ala Met Glu Met Tyr Arg Gln Lys Arg Leu Ala Glu Trp
 65                  70                  75                  80

aaa gct aac cag atg aag aat gtt ttc ggg gag ctg aag gag atc tcg   288
Lys Ala Asn Gln Met Lys Asn Val Phe Gly Glu Leu Lys Glu Ile Ser
                 85                  90                  95

ggg cag gat tac gtg cag gag gtc aat aaa gct gga gag gga atc tgg   336
Gly Gln Asp Tyr Val Gln Glu Val Asn Lys Ala Gly Glu Gly Ile Trp
            100                 105                 110

gtg gtg ctg cac ctc tac aaa caa ggg atc ccg ctg tgt tcg ctc att   384
Val Val Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys Ser Leu Ile
        115                 120                 125

aat cag cat ctg gct caa ctg gcc cgc aag ttt cct cag agt aag ttc   432
Asn Gln His Leu Ala Gln Leu Ala Arg Lys Phe Pro Gln Ser Lys Phe
    130                 135                 140

ctg aag tcc atc tcc agc acc tgc atc ccc aac tac ccc gac cgc aac   480
Leu Lys Ser Ile Ser Ser Thr Cys Ile Pro Asn Tyr Pro Asp Arg Asn
145                 150                 155                 160

ctg ccc acg ctg ttc gtg tac cgc gac gga gag atg aag gcg cag ttc   528
Leu Pro Thr Leu Phe Val Tyr Arg Asp Gly Glu Met Lys Ala Gln Phe
                165                 170                 175
```

```
atc ggc ccg ctg gtg ttc gga ggg atg aac ctc acc tgt gac gag ctg   576
Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Cys Asp Glu Leu
            180                 185                 190

gag tgg cgt ctg tca gag tct gga gct gtg aag aca gat ctg gag gaa   624
Glu Trp Arg Leu Ser Glu Ser Gly Ala Val Lys Thr Asp Leu Glu Glu
        195                 200                 205

aac ccc aga aaa cag atc cag gat cag ctg atg acg tcc att cgc tgc   672
Asn Pro Arg Lys Gln Ile Gln Asp Gln Leu Met Thr Ser Ile Arg Cys
    210                 215                 220

tcg gca aac aca cac cga gac gga gag gaa gac tct gat gaa gac tga   720
Ser Ala Asn Thr His Arg Asp Gly Glu Glu Asp Ser Asp Glu Asp
225                 230                 235                 240
```

<210> 6
<211> 239
<212> PRT
<213> Danio rerio

<400> 6

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Met Gln Asp Pro Asn Ala Asp Thr Glu Trp Asn Asp Ile Leu Arg Lys
  1               5                  10                  15
Lys Gly Ile Leu Pro Pro Lys Glu Thr Pro Val Glu Glu Glu Glu Asp
             20                  25                  30
Glu Gln Leu His Leu Gln Ser Gln Ser Val Val Lys Thr Tyr Glu Asp
         35                  40                  45
Met Thr Leu Glu Glu Leu Glu Glu Asn Glu Asp Glu Phe Ser Glu Glu
     50                  55                  60
Asp Glu His Ala Met Glu Met Tyr Arg Gln Lys Arg Leu Ala Glu Trp
 65                  70                  75                  80
Lys Ala Asn Gln Met Lys Asn Val Phe Gly Glu Leu Lys Glu Ile Ser
                 85                  90                  95
Gly Gln Asp Tyr Val Gln Glu Val Asn Lys Ala Gly Glu Gly Ile Trp
            100                 105                 110
Val Val Leu His Leu Tyr Lys Gln Gly Ile Pro Leu Cys Ser Leu Ile
        115                 120                 125
Asn Gln His Leu Ala Gln Leu Ala Arg Lys Phe Pro Gln Ser Lys Phe
    130                 135                 140
Leu Lys Ser Ile Ser Ser Thr Cys Ile Pro Asn Tyr Pro Asp Arg Asn
145                 150                 155                 160
Leu Pro Thr Leu Phe Val Tyr Arg Asp Gly Glu Met Lys Ala Gln Phe
                165                 170                 175
Ile Gly Pro Leu Val Phe Gly Gly Met Asn Leu Thr Cys Asp Glu Leu
            180                 185                 190
Glu Trp Arg Leu Ser Glu Ser Gly Ala Val Lys Thr Asp Leu Glu Glu
        195                 200                 205
Asn Pro Arg Lys Gln Ile Gln Asp Gln Leu Met Thr Ser Ile Arg Cys
    210                 215                 220
Ser Ala Asn Thr His Arg Asp Gly Glu Glu Asp Ser Asp Glu Asp
225                 230                 235
```

<210> 7
<211> 723
<212> DNA
<213> Drosophila melanogaster

<220>
<221> CDS
<222> (1)..(723)

<400> 7

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atg cag gac cca aac gaa gat acc gag tgg aat gat gtg ctc cga gcc   48
Met Gln Asp Pro Asn Glu Asp Thr Glu Trp Asn Asp Val Leu Arg Ala
  1               5                  10                  15

aag gga ata att ggg ccc aag gcg aag gag gcg gag atc aca gag gat   96
Lys Gly Ile Ile Gly Pro Lys Ala Lys Glu Ala Glu Ile Thr Glu Asp
             20                  25                  30

cag atc cag aag ctg atg gac gat gcc atc cag cgg cgc aca gat ctg   144
Gln Ile Gln Lys Leu Met Asp Asp Ala Ile Gln Arg Arg Thr Asp Leu
         35                  40                  45

cca ctg aat gaa ggc cag cgc gac aag aag atc gac gac atg tcg ctg   192
Pro Leu Asn Glu Gly Gln Arg Asp Lys Lys Ile Asp Asp Met Ser Leu
     50                  55                  60

gac gaa ctc gac gaa ctg gag gat tcc gag gac gag gct gtt cta gag   240
Asp Glu Leu Asp Glu Leu Glu Asp Ser Glu Asp Glu Ala Val Leu Glu
 65                  70                  75                  80

cag tat cgc cag cga cgc atc gcc gag atg agg gcc acc gct gaa aag   288
Gln Tyr Arg Gln Arg Arg Ile Ala Glu Met Arg Ala Thr Ala Glu Lys
                 85                  90                  95

gcg cga ttt gga tca gtg cgc gag atc tca gga cag gat tat gtc aac   336
Ala Arg Phe Gly Ser Val Arg Glu Ile Ser Gly Gln Asp Tyr Val Asn
            100                 105                 110

gag gtg acc aag gcc ggc gag ggc atc tgg gtg gta ctc cac ctg tat   384
Glu Val Thr Lys Ala Gly Glu Gly Ile Trp Val Val Leu His Leu Tyr
        115                 120                 125

gcc aac ggc gta ccg ctg tgc gca ctg atc cac cat cac atg cag cag   432
Ala Asn Gly Val Pro Leu Cys Ala Leu Ile His His His Met Gln Gln
    130                 135                 140

ctg gcc gtc cgc ttt cca cag acc aag ttc gtg tgc tcc gtt gcc acc   480
Leu Ala Val Arg Phe Pro Gln Thr Lys Phe Val Cys Ser Val Ala Thr
145                 150                 155                 160

acc tgc ata cca aac ttc ccc gag aag aac ctg ccc acc atc ttt atc   528
Thr Cys Ile Pro Asn Phe Pro Glu Lys Asn Leu Pro Thr Ile Phe Ile
                165                 170                 175

tac cac gag ggt gcg ctg cgc aag cag tac ata ggc cca ctg gag ctg   576
Tyr His Glu Gly Ala Leu Arg Lys Gln Tyr Ile Gly Pro Leu Glu Leu
            180                 185                 190

cgc ggc gac aag ttg acc gct gag gag ctg gag ttt atg ctg ggt cag   624
Arg Gly Asp Lys Leu Thr Ala Glu Glu Leu Glu Phe Met Leu Gly Gln
        195                 200                 205

gcg gga gca gtg ccc acc gag atc acg gag gat cca cgg ccg cag atc   672
Ala Gly Ala Val Pro Thr Glu Ile Thr Glu Asp Pro Arg Pro Gln Ile
    210                 215                 220
```

```
agg gac aag atg ctt gcc gat ctc gaa gac aaa agc tcg gac ttc tac   720
Arg Asp Lys Met Leu Ala Asp Leu Glu Asp Lys Ser Ser Asp Phe Tyr
225                 230                 235                 240

tga                                                               723
```

<210> 8
<211> 240
<212> PRT
<213> Drosophila melanogaster

<400> 8

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Met Gln Asp Pro Asn Glu Asp Thr Glu Trp Asn Asp Val Leu Arg Ala
  1               5                  10                  15
Lys Gly Ile Ile Gly Pro Lys Ala Lys Glu Ala Glu Ile Thr Glu Asp
            20                  25                  30
Gln Ile Gln Lys Leu Met Asp Asp Ala Ile Gln Arg Arg Thr Asp Leu
        35                  40                  45
Pro Leu Asn Glu Gly Gln Arg Asp Lys Lys Ile Asp Asp Met Ser Leu
    50                  55                  60
Asp Glu Leu Asp Glu Leu Glu Asp Ser Glu Asp Glu Ala Val Leu Glu
65                  70                  75                  80
Gln Tyr Arg Gln Arg Arg Ile Ala Glu Met Arg Ala Thr Ala Glu Lys
                85                  90                  95
Ala Arg Phe Gly Ser Val Arg Glu Ile Ser Gly Gln Asp Tyr Val Asn
            100                 105                 110
Glu Val Thr Lys Ala Gly Glu Gly Ile Trp Val Val Leu His Leu Tyr
        115                 120                 125
Ala Asn Gly Val Pro Leu Cys Ala Leu Ile His His His Met Gln Gln
    130                 135                 140
Leu Ala Val Arg Phe Pro Gln Thr Lys Phe Val Cys Ser Val Ala Thr
145                 150                 155                 160
Thr Cys Ile Pro Asn Phe Pro Glu Lys Asn Leu Pro Thr Ile Phe Ile
                165                 170                 175
Tyr His Glu Gly Ala Leu Arg Lys Gln Tyr Ile Gly Pro Leu Glu Leu
            180                 185                 190
Arg Gly Asp Lys Leu Thr Ala Glu Glu Leu Glu Phe Met Leu Gly Gln
        195                 200                 205
Ala Gly Ala Val Pro Thr Glu Ile Thr Glu Asp Pro Arg Pro Gln Ile
    210                 215                 220
Arg Asp Lys Met Leu Ala Asp Leu Glu Asp Lys Ser Ser Asp Phe Tyr
225                 230                 235                 240
```

<210> 9
<211> 861
<212> DNA
<213> Saccharomyces cerevisiae

<220>
<221> CDS
<222> (1)..(861)

<400> 9

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atg cag aat gaa cca atg ttt cag gtc cag gtg gac gaa tct gaa gac   48
Met Gln Asn Glu Pro Met Phe Gln Val Gln Val Asp Glu Ser Glu Asp
  1               5                  10                  15
```

```
agt gaa tgg aac gat att tta aga gcg aag ggt gta ata cca gaa cgt   96
Ser Glu Trp Asn Asp Ile Leu Arg Ala Lys Gly Val Ile Pro Glu Arg
            20                  25                  30

gca cct tcg ccc act gca aag tta gaa gaa gca tta gaa gaa gca att  144
Ala Pro Ser Pro Thr Ala Lys Leu Glu Glu Ala Leu Glu Glu Ala Ile
        35                  40                  45

gcc aag cag cat gaa aat aga cta gaa gat aaa gac ttg tcg gat ttg  192
Ala Lys Gln His Glu Asn Arg Leu Glu Asp Lys Asp Leu Ser Asp Leu
    50                  55                  60

gaa gaa cta gaa gac gat gaa gat gaa gat ttc ttg gaa gct tac aag  240
Glu Glu Leu Glu Asp Asp Glu Asp Glu Asp Phe Leu Glu Ala Tyr Lys
65                  70                  75                  80

atc aaa aga tta aat gaa atc cgc aaa tta cag gaa cgt tcc aaa ttt  288
Ile Lys Arg Leu Asn Glu Ile Arg Lys Leu Gln Glu Arg Ser Lys Phe
                85                  90                  95

gga gaa gtt ttc cac att aac aaa cct gaa tac aac aaa gag gtt act  336
Gly Glu Val Phe His Ile Asn Lys Pro Glu Tyr Asn Lys Glu Val Thr
            100                 105                 110

ttg gcc agt cag gga aag aaa tat gaa ggt gca caa acc aat gac aat  384
Leu Ala Ser Gln Gly Lys Lys Tyr Glu Gly Ala Gln Thr Asn Asp Asn
        115                 120                 125

ggt gaa gag gat gac ggt ggt gtc tac gta ttc gtt cat ctc tcg ctt  432
Gly Glu Glu Asp Asp Gly Gly Val Tyr Val Phe Val His Leu Ser Leu
    130                 135                 140

caa agt aaa cta caa agc aga att ctg tct cat ctt ttc caa tct gct  480
Gln Ser Lys Leu Gln Ser Arg Ile Leu Ser His Leu Phe Gln Ser Ala
145                 150                 155                 160

gca tgc aaa ttc aga gaa ata aaa ttt gta gaa ata cct gcc aat aga  528
Ala Cys Lys Phe Arg Glu Ile Lys Phe Val Glu Ile Pro Ala Asn Arg
                165                 170                 175

gca att gaa aac tat ccc gaa tcc aat tgc ccg aca tta att gta tat  576
Ala Ile Glu Asn Tyr Pro Glu Ser Asn Cys Pro Thr Leu Ile Val Tyr
            180                 185                 190

tac cgg ggt gag gta atc aaa aac atg ata acg cta cta gaa ctg ggt  624
Tyr Arg Gly Glu Val Ile Lys Asn Met Ile Thr Leu Leu Glu Leu Gly
        195                 200                 205

ggt aat aat tcc aag atg gaa gac ttt gaa gat ttt atg gta aaa gtt  672
Gly Asn Asn Ser Lys Met Glu Asp Phe Glu Asp Phe Met Val Lys Val
    210                 215                 220

ggc gct gtt gca gaa gga gac aac aga ctg ata atg aac cga gac gat  720
Gly Ala Val Ala Glu Gly Asp Asn Arg Leu Ile Met Asn Arg Asp Asp
225                 230                 235                 240

gaa gaa tcc agg gaa gag aga aaa ttg cat tac ggt gaa aaa aaa tcg  768
Glu Glu Ser Arg Glu Glu Arg Lys Leu His Tyr Gly Glu Lys Lys Ser
                245                 250                 255
```

```
atc agg tca ggt att aga gga aaa ttt aat gtc ggc ata ggt gga aat   816
Ile Arg Ser Gly Ile Arg Gly Lys Phe Asn Val Gly Ile Gly Gly Asn
            260                 265                 270

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         35                  40                  45
Ala Lys Gln His Glu Asn Arg Leu Glu Asp Lys Asp Leu Ser Asp Leu
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Ile Lys Arg Leu Asn Glu Ile Arg Lys Leu Gln Glu Arg Ser Lys Phe
                 85                  90                  95
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            100                 105                 110
Leu Ala Ser Gln Gly Lys Lys Tyr Glu Gly Ala Gln Thr Asn Asp Asn
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/20576

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/11 C07K14/47 C07K16/18 C12Q1/68
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, EMBL, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUZUKI ATSUSHI ET AL: "Resistance to Fas-mediated apoptosis: Activation of capase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP." ONCOGENE, vol. 17, no. 8, August 1998 (1998-08), pages 931-939, XP000979228 ISSN: 0950-9232 abstract --- -/--	12-14

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2001	14/02/2001

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/20576

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! 28 January 1998 (1998-01-28) MARRA M., ET AL.: "similar to TR:Q12017 Q12017 HYPOTHETICAL 32.8 KD PROTEIN." retrieved from EBI Database accession no. AA763356 XP002158477 abstract	1,3, 6-13, 15-17, 19, 21-27, 29-41, 56-59
X	DATABASE EMBL 'Online! 1 February 1999 (1999-02-01) HARVEY D. ET AL.: "Drosophila melanogaster cDNA clone GH20282 5prime, mRNA sequence." retrieved from EBI Database accession no. AI389174 XP002158481 abstract	1,5-12, 15-17, 19, 21-27, 29-41, 56-59
A	DATABASE EMBL 'Online! 7 January 1999 (1999-01-07) MATTHEWS L.: "Human DNA sequence from clone 44A20 on chromosome 6q23.1-24.3" retrieved from EBI Database accession no. AL035086 XP002158478 abstract	2,6-13, 15-18, 21-27, 29-41, 56-59
A	DATABASE TREMBLREL 'Online! 1 November 1999 (1999-11-01) WILSBACHER L.D. ET AL.: "The mouse Clock locus: Sequence and analysis of 204 kb from mouse . TPHLP (FRAGMENT)" retrieved from EBI Database accession no. Q9WUP3 XP002158479 abstract	1-27, 29-41, 56-59
A	DATABASE EMBL 'Online! 16 March 1999 (1999-03-16) CLARK M. ET AL.: "Q12017 HYPOTHETICAL 32.8 KD PROTEIN. ; mRNA sequence." retrieved from EBI Database accession no. AI437036 XP002158480 abstract	1-27, 29-41, 56-59

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: CLONING AND CHARACTERIZATION OF VIRAL IAP ASSOCIATED FACTOR (VIAF) IN SEVERAL ORGANISMS

(57) Abstract: DNA and proteins sequences are disclosed for several VIAF homologs. The VIAF sequence modulates the anti-apoptotic and signaling properties of IAP family members. The disclosure also includes specific binding agents (such as antibodies) that recognize VIAF, methods of decreasing apoptosis by increasing VIAF expression, methods of increasing apoptosis by decreasing VIAF expression, methods of treating disease caused by mutations, therapeutic compositions that include VIAF, recombinant DNA molecules, probes, and transformed cells that incorporate the DNA sequence to express VIAF. The disclosure also includes methods of diagnosis and treatment of diseases caused by an increased expression of VIAF, such as neurodegenerative diseases, and methods of treatment of diseases caused by unwanted apoptosis, such as occurs in autoimmune diseases and neurodegenerative disorders. The disclosure also includes methods of diagnosis and treatment of diseases caused by an underexpression of VIAF, such as cancer.

CLONING AND CHARACTERIZATION OF VIRAL IAP ASSOCIATED FACTOR (VIAF) IN SEVERAL ORGANISMS

FIELD

This disclosure relates to nucleic acid and amino acid sequences corresponding to VIAF genes in several species. The sequences are useful for modulating the anti-apoptotic and signaling properties of the IAP family.

BACKGROUND

The process of apoptosis, or programmed cell death, can be utilized to eliminate unwanted cells (Kerr et al., 1972, *Br. J. Cancer* 4:239-57). This can occur during embryogenesis, turnover of senescent cells or metamorphosis. It also represents a very efficient defense mechanism against pathogens, such as viruses, by allowing the host organism to eliminate infected cells. However, unwanted apoptosis can also occur, for example in pathological conditions associated with autoimmunity, AIDS, and retinal degeneration.

In response to this defense mechanism, viruses such as herpesviruses, poxviruses and insect baculoviruses, have developed counter-strategies to escape or retard the apoptosis triggered by the host organism (Henderson et al, 1993, *Proc. Natl. Acad. Sci. U S A.* 90:8479-83; Cheng et al.,1997, *Science* 278:1966-8). An example of such a viral mechanism is the expression of proteins that inhibit or regulate apoptosis. Several viral proteins that inhibit apoptosis have been identified (Eiben and Duckett, 1998, *Results Probl. Cell. Differ.* 24:91-104). One group of inhibitory proteins, such as the cowpox serpin CrmA or the baculoviral p35 gene product, act as pseudosubstrate inhibitors of one or more caspases, the known mediators of apoptosis. While CrmA inhibits only caspase-1 and caspase-8, p35 is a more broad-spectrum caspase inhibitor. These viral gene products block proteolytically active caspases by remaining tightly bound to the active site following their proteolysis.

In contrast, other baculoviruses such as *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpMPNPV) or *Cydia pomonella* granulosis virus (CpGV) do not express p35. Instead, they express the family of inhibitors of apoptosis proteins (IAP) (Clem and Miller, 1994, *J. Virol.* 67:3730-8). The IAPs have been implicated in a variety of essential cellular processes in addition to apoptosis, including signal transduction, cell cycle regulation, control of gene transcription and ubiquitination (Rothe *et al.*, 1995, *Cell* 83:1243-52; Chu *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:10057-62; Sanna *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95:6015-20). Expresson of Op-IAP in mammalian cells has been shown to confer protection from a variety of apoptotic stimuli including Fas/Apo-1/CD95 (Duckett *et al.* 1996, *EMBO J.* 15:2685-94; Hawkins *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93:13786-90; Hawkins *et al.*, 1998, *Cell. Death Differ.* 5:569-76). In insect cells IAPs have been shown to bind to the apoptotic inducers Reaper, Hid, Grim and Doom, suggesting that their protective effects may be mediated through multiple targets (Harvey *et al.*,

1996, *Mol. Cell. Biol.* 17:2835-43; Manji *et al.*, 1997, *J. Virol.* 71: 4509-16; Vucic *et al.* 1998, *Mol. Cell. Biol.* 18:3300-9). However, in mammals no analogous proteins have been identified.

The structure of the baculoviral IAPs is characterized by two types of sequence motifs. The C-terminus features a zinc binding domain known as a RING-finger, and the N-terminus exhibits one to three approximately 65 amino acid long Cys/His-rich sequences termed baculovirus IAP repeat (BIR). Both the RING finger and BIR repeats have been shown to be essential for preventing apoptosis. It has also been shown that the human homologue hILP/XIAP is a potent inhibitor of the effector caspase-3 but does not inhibit proteolytically active caspase-1,-6 or -8. This inhibition is mediated by the BIR motifs of the protein whereas the RING finger was not necessary for this interaction. Two other cellular IAPs, c-IAP1 and c-IAP2, are components of the type 2 tumor necrosis factor receptor cytoplasmic complex and shown to be required to suppress TNF-α induced apoptosis.

SUMMARY

Although the mechanism by which mammalian IAPs regulate apoptosis has been investigated, the mechanism of their action remains unknown. To gain an understanding of the mechanism used by IAPs to antagonize apoptosis, the identification of proteins which interact with the IAPs would be useful.

Herein disclosed is a novel protein, viral IAP-associated factor (VIAF), that is evolutionary conserved across several species and interacts directly with baculoviral OpIAP as well as mammalian XIAP, c-IAP1 and c-IAP2. VIAF modulates the anti-apoptotic and signaling properties of the IAP family. For example, VIAF inhibits Bax- and Fas-induced apoptosis and co-expression of VIAF with suboptimal quantities of XIAP confers almost complete protection. In certain disclosed embodiments, VIAF and XIAP synergistically stimulates c-Jun N-terminal kinase activity.

The cDNA and protein sequence of VIAF, for several different organisms including human, mouse, zebrafish, and *Drosophila*, are also disclosed.

Disclosed herein is a purified protein having VIAF biological activity, which modulates the anti-apoptotic and signaling properties of the IAP family. In some disclosed embodiments, the VIAF protein has the amino acid sequence shown in either SEQ ID NOS 2, 4, 6, or 8 or amino acid sequences that differ from those specified in SEQ ID NOS 2, 4, 6, or 8 by one or more conservative amino acid substitutions, or amino acid sequences having at least 80% sequence identity to those sequences, for example sequences that are at least 85%, 90%, 95% or even 98% or 99% identical. Other embodiments include a VIAF protein containing no more than about 240 amino acid residues, or in other embodiments, no more than about 250, 260, or 270 residues. In other embodiments, the amino acid sequence contains at least 10, 15, 20, or 25 contiguous amino acid residues of SEQ ID NOS 2, 4, 6, or 8. Also included is an isolated nucleic acid molecule

encoding a biologically active VIAF protein, particularly such molecules that include a promoter sequence operably linked to the nucleic acid molecule for expression of the VIAF protein, as well as transgenic cells containing these molecules. In addition to such variants that retain biological activity of the VIAF protein, fragments of the sequences that have or retain such activity may be used. Such fragments may, for example, include at least 50%, 75%, 90% or 95% of the amino acid residues of the native peptide sequence.

Also disclosed are animal proteins having VIAF biological activity. In particular embodiments, the animal is a higher eukaryote, for example a mammal. In yet other embodiments, the mammal is a mouse or human.

In particular embodiments, the isolated nucleic acid molecule includes at least 21 contiguous nucleotides of a sequence selected from SEQ ID NOS 3 or 7 or its complementary strand. In other embodiments, the isolated nucleic acid molecule includes at least 22 contiguous nucleotides of a sequence selected from SEQ ID NO 5 or its complementary strand. Alternatively, the isolated nucleic acid molecule includes at least 30 or 50 contiguous nucleotides of SEQ ID NOS 3, 5, or 7, or a nucleic acid molecule that is at least 80% homologous to SEQ ID NOS 1, 3, 5, or 7, and encodes a protein having VIAF biological activity. Alternatively, the nucleic acid molecule has a sequence which hybridizes under stringent conditions to the sequences defined in SEQ ID NOS 1, 3, 5, or 7, or which has the full length sequence of SEQ ID NOS 1, 3, 5, or 7 or its complementary strand. In other embodiments, the nucleic acid molecule has a sequence which hybridizes under conditions of at least 75% or 90% stringency to the sequences defined in SEQ ID NO NOS 1, 3, 5, or 7, or which has the full length sequence of SEQ ID NOS 1, 3, 5, or 7 or its complementary strand. In yet another embodiment, the nucleic acid molecule has a sequence depicted as bases 62-781 of SEQ ID NO 1, and encodes a protein having VIAF biological activity, for example the amino acid sequence shown in SEQ ID NO 2.

Another embodiment includes an isolated nucleic acid molecule which includes at least 370 contiguous nucleotides of a nucleic acid sequence shown in SEQ ID NO 1, or its complementary strand, or 20, 30, 50 or more contiguous nucleotides from nucleotides 62-334 of SEQ ID NO 1, or its complementary strand. The 20, 30 or 50 contiguous nucleotides can alternatively be taken from nucleotides 1-334 or 704-781, or 62-334 or 703-781, of SEQ ID NO 1, or its complementary strand.

Another embodiment includes isolated nucleic acid molecules (such as oligonucleotides) which are capable of specifically hybridizing to a VIAF gene, for example a nucleic acid molecule having at least 25 consecutive nucleotides of the sequences shown in SEQ ID NOS 3, 5, or 7. Alternatively, the nucleic acid molecules have at least 21, 30, or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 3; at least 22, 30 or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 5; or at least 21, 30 or 50 contiguous nucleotides of the sequences shown in SEQ ID NO 7. In yet another embodiment, antisense oligonucleotides are disclosed which

hybridize to RNA or a plus strand of the nucleic acids disclosed herein and inhibits VIAF biological activity are provided.

Also disclosed herein are recombinant vectors that include any of the nucleic acid molecules, and transgenic hosts into which the recombinant vector is incorporated. Also disclosed are the purified peptides encoded by any of these nucleic acid molecules, such as proteins (including fusion proteins) having VIAF biological activity which can be used to modulate the anti-apoptotic and signaling properties of the IAP family. In particular embodiments, the peptide has an amino acid sequence shown in SEQ ID NO 2, 4, 6, or 8, or variants or fragments thereof.

Also disclosed are specific binding agents capable of specifically binding to a VIAF protein, for example polyclonal antibodies, monoclonal antibodies, and fragments of monoclonal antibodies that specifically bind to the VIAF protein. Such specific binding agents can be used in assays for quantitating amounts of purified VIAF, for example to diagnose diseases associated with abnormal VIAF expression.

Other embodiments include a composition having a therapeutically effective amount of a protein with VIAF biological activity, in combination with a pharmaceutically acceptable carrier, for treating conditions in which VIAF activity is impaired or lost. In other embodiments (examples), the composition further includes one or more other anti-apoptotic compounds. The protein having VIAF biological activity contained within such compositions includes any VIAF protein or peptide disclosed herein, including fragments and variants. In other examples, the composition can include a therapeutically effective amount of a specific binding agent described above and a pharmaceutically acceptable carrier. In yet other examples, the composition can include a therapeutically effective amount of antisense oligonucleotides described above and a pharmaceutically acceptable carrier.

The disclosed compositions can be used to decrease apoptosis. In other embodiments, the compositions disclosed herein can be used to inhibit Bax- and Fas-induced apoptosis, for example in amounts sufficient to inhibit Bax- and Fas-induced apoptosis in a subject, such as a human, who suffers from unwanted apoptosis. The compositions can be used in subjects who suffer from a condition such as a cancer, autoimmune or neurodegenerative disease, for example diabetes, multiple sclerosis or retinal degeneration, which is characterized by unwanted apoptosis. Alternatively, the disclosed compositions can be used for promoting apoptosis in a subject, such as a human, in whom apoptosis is desired. The compositions can be used in subjects who suffer from a condition such as cancer.

Also disclosed herein are methods for detecting an enhanced susceptibility of a subject to disease associated with abnormal apoptosis, by detecting a deletion of or within a VIAF gene, detecting other mutations of a VIAF gene and/or the abnormal expression such as a decrease or absence of VIAF protein in cells of a subject, such as a human. For example, in an extreme case, a total absence of VIAF protein may be detected. The disease may be cancer in which apoptosis is

abnormally decreased, or an autoimmune disease, or a neurodegenerative disease in which apoptosis is abnormally increased. In certain embodiments, a mutation (such as a substitution, insertion or deletion) of or in a VIAF gene can be detected by incubating a nucleic acid, such as an oligonucleotide, with the nucleic acid of the cell under conditions such that the oligonucleotide will specifically hybridize to a VIAF gene present in the nucleic acid to form an oligonucleotide:VIAF gene complex, and then detecting an increase or decrease of oligonucleotide:VIAF complexes, wherein the decrease of said complexes indicates a mutation (such as a deletion of or within) the VIAF gene. In an extreme case, this mutation may be a total absence of the VIAF gene. The present invention also provides methods for detecting the presence of VIAF protein in a cell by incubating a specific binding agent of the present invention with proteins of the cell under conditions such that the specific binding agent will specifically bind to a VIAF protein present in the cell to form a specific binding agent:VIAF protein complex, and detecting an increase or decrease (or quantity) of specific binding agent:VIAF protein complexes, including a total absence of the VIAF protein.

In another embodiment, VIAF biological activity can be supplied to a cell which has lost its VIAF biological activity, for example by a mutation of the VIAF gene, for example by a deletion of all or a portion of a VIAF gene, by introducing a VIAF gene into the cell so that the VIAF gene is expressed in the cell. In specific embodiments, the VIAF biological activity is supplied to treat a disease of abnormal apoptosis.

Also disclosed herein are methods for decreasing apoptosis in a cell by increasing the level of VIAF biological activity, which prevents the cell from undergoing apoptosis. In one embodiment, the cell is a neuron which has decreased VIAF protein expression or the cell has decreased VIAF biological activity or expression, relative to VIAF biological activity or expression in a same tissue type that is undergoing apoptosis. In certain embodiments, increasing the level of VIAF biological activity can be achieved by exposing the cell to a therapeutically effective amount of any of the VIAF proteins (including fragments and variants) disclosed herein. In other embodiments, the methods for decreasing apoptosis can be used to treat a disease of abnormal apoptosis, for example, autoimmune and neurodegenerative diseases, such as diabetes, multiple sclerosis and retinal degeneration.

Methods are also provided for inducing apoptosis in a subject by administering a therapeutically effective amount of an antisense oligonucleotide disclosed herein sufficient to induce apoptosis (including increased apoptosis) in the subject, for example a human. In a particular embodiment, inducing apoptosis treats a disease of insufficient apoptosis. In another embodiment, the antisense oligonucleotide specifically inhibits expression of VIAF protein, for example for diseases associted with excess apoptosis. The antisense molecule may include at least 20 contiguous nucleotides of a sequence that is complementary to at least a portion of an RNA

transcript of a VIAF gene, and is hybridizable to the RNA transcript, such as SEQ ID NOs 1, 3, 5 or 7.

In another embodiment, methods for treating a disease caused by a mutation in SEQ ID NOS 1, 3, 5, or 7, or a complementary strand, by supplying a therapeutically effective amount of a polypeptide product or the nucleic acid, are disclosed.

In yet another embodiment, a method is disclosed of inhibiting Bax- and Fas-induced apoptosis in a subject by administering a therapeutically effective amount of a purified protein having VIAF biological activity, or a nucleic acid which can express a protein having VIAF biological activity. In particular embodiments, the purified protein is any protein disclosed herein, which has VIAF biological activity.

The foregoing and other objects, features, and advantages disclosed herein will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the protein sequence of a human VIAF and alignment with sequences of some VIAF homologs in other species. Identical amino acids are shaded in gray.

FIG. 2 shows a predicted structure for a human VIAF protein having the amino acid sequence shown in FIG 1.

FIG. 3 is a digital image of a Northern blot showing VIAF mRNA expression in several tissues. As a control, the blot was probed for β-actin (lower panel).

FIG. 4 is a digital image of a dried SDS-polyacrylamide gel, showing the results of a precipitation experiment demonstrating that VIAF associates with Op-IAP *in vitro*.

FIG. 5 is a digital image of a western blot showing that endogenous VIAF and XIAP proteins interact *in vivo*.

FIG. 6 is a digital image of a western blot showing that the BIR domain of XIAP is necessary and sufficient for association with VIAF. The *in vitro* translated protein prior to precipitation is shown as input lanes.

FIG. 7 is a digital image of a western blot showing that VIAF interacts with XIAP, c-IAP1 and c-IAP2.

FIG. 8A is a graph showing that VIAF protects from Bax-induced apoptosis. Results shown are representative of three independent experiments.

FIG. 8B is a graph showing that VIAF protects from and Fas/Apo-1/CD95-induced apoptosis. Results shown are representative of three independent experiments.

FIG. 9 is a digital image of protein gels, and a bar graph quantitating the image, showing the synergistic effect of VIAF and XIAP on JNK activation.

FIG. 10 is a digital image of a Western blot showing that dexamethasone-induced VIAF expression in murine thymocytes is abrogated by ZVAD-fmk.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO 1 shows a nucleotide sequence of a human VIAF cDNA, GenBank Accession No. AF110511.

SEQ ID NO 2 shows an amino acid sequence of a human VIAF protein, GenBank Accession No. AF110511.

SEQ ID NO 3 shows a nucleotide sequence of a mouse VIAF cDNA, GenBank Accession No. AF110512.

SEQ ID NO 4 shows an amino acid sequence of a mouse VIAF protein, GenBank Accession No. AF110512.

SEQ ID NO 5 shows a nucleotide sequence of a Zebrafish VIAF cDNA, GenBank Accession No. AF175204.

SEQ ID NO 6 shows an amino acid sequence a Zebrafish VIAF protein, GenBank Accession No. AF175204.

SEQ ID NO 7 shows a nucleotide sequence of a *Drosophila* VIAF cDNA, GenBank Accession No. AF110513.

SEQ ID NO 8 shows an amino acid sequence a *Drosophila* VIAF protein, GenBank Accession No. AF110513.

SEQ ID NO 9 shows a nucleotide sequence of a *S. cerevisiae* VIAF cDNA, GenBank Accession No. AF110514.

SEQ ID NO 10 shows an amino acid sequence a *S. cerevisiae* VIAF protein, GenBank Accession No. AF110514.

SEQ ID NO 11 shows a nucleotide sequence of a sense primer that can be used to amplify the human VIAF cDNA.

SEQ ID NO 12 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the human VIAF cDNA.

SEQ ID NO 13 shows a nucleotide sequence of a sense primer that can be used to amplify the mouse VIAF cDNA.

SEQ ID NO 14 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the mouse VIAF cDNA.

SEQ ID NO 15 shows a nucleotide sequence of a sense primer that can be used to amplify the zebrafish VIAF cDNA.

SEQ ID NO 16 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the zebrafish VIAF cDNA.

SEQ ID NO 17 shows a nucleotide sequence of a sense primer that can be used to amplify the *Drosophila* VIAF cDNA.

SEQ ID NO 18 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the *Drosophila* VIAF cDNA.

SEQ ID NO 19 shows a nucleotide sequence of a sense primer that can be used to amplify the *S. cerevisiae* VIAF cDNA.

SEQ ID NO 20 shows a nucleotide sequence of an anti-sense primer that can be used to amplify the *S. cerevisiae* VIAF cDNA.

SEQ ID NO 21 shows an amino acid sequence that can be used to generate a polyclonal antibody that recognizes the N-terminal region of VIAF.

SEQ ID NO 22 shows an amino acid sequence that can used to generate a polyclonal antibody that recognizes the C-terminal region of VIAF.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Abbreviations and Definitions

The following definitions and methods are provided to better define the materials and methods disclosed herein and to guide those of ordinary skill in the art in the practice of the materials and methods disclosed herein. As used herein (including the appended claims), the singular forms "a" or "an" or "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protein" includes a plurality of such proteins and reference to "the antibody" includes reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abnormal apoptosis: An amount of apoptosis, for example too much, too little, or both. For example, the amount of apoptosis associated with a disease of abnormal apoptosis can be compared with normal cells having normal apoptosis, for example cells of the same tissue type which do not have the disease. The amount of apoptosis can be determined using the methods described in EXAMPLE 9.

Animal protein: A higher eukaryotic protein, for example a protein expressed in mammals, fish, and *Drosophila*. The animal protein can be isolated from the animal, or expressed

recombinantly, for example in bacteria. Excludes lower eukaryotic proteins, for example, yeast, for example *S. cerevisiae*.

Antisense molecules or antisense oligonucleotides: Nucleic acid molecules that are specifically hybridizable or specifically complementary to either RNA or the plus strand of DNA (Weintraub, *Scientific American* 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double stranded. In one embodiment, the antisense oligomer is about 15 nucleotides, which are easily synthesized. The use of antisense molecules to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.* 172:289, 1988).

Therapeutically effective antisense molecules are characterized by their ability to inhibit the expression of VIAF. Complete inhibition is not necessary for therapeutic effectiveness, some oligonucleotides will be capable of inhibiting the expression of VIAF by at least 15%, 30%, 40%, 50%, 60%, or 70%.

Therapeutically effective antisense molecules are additionally characterized by being sufficiently complementary to VIAF encoding nucleic acid sequences. As described below, sufficient complementary means that the therapeutically effective oligonucleotide or oligonucleotide analog can specifically disrupt the expression of VIAF, and not significantly alter the expression of genes other than VIAF.

Calpain Inhibitor I: An inhibitor of calpain I, II, cathepsin B and cathepsin L. It inhibits: neutral cysteine proteases and the proteasome; apoptosis in thymocytes; and the proteolysis of IκBα and IκBα by the ubiquitin-proteasome complex.

Cancer: Malignant neoplasm that has undergone characteristic anaplasia with loss of differentiation, increased rate of growth, invasion of surrounding tissue, and is capable of metastasis.

cDNA (complementary DNA): A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted from cells.

Chemical synthesis: The artificial means by which one can make a protein or peptide, for example as described in EXAMPLE 23.

Deletion: The removal of a sequence of DNA, the regions on either side being joined together.

DNA: Deoxyribonucleic acid. DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules

code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

Isolated: An "isolated" biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Lactacystin: A cell-permeable and irreversible proteasome inhibitor. It is a *Streptomyces* metabolite that acts as a highly specific inhibitor of the 20S proteasome, and blocks proteasome activity by targeting the catalytic β-subunit.

Malignant: Cells which have the properties of anaplasia invasion and metastasis.

Mimetic: A molecule (such as an organic chemical compound) that mimics the activity of a protein, such as the biological activity of VIAF. Peptidomimetic and organomimetic embodiments are within the scope of this term, wherein the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid sidechains in the peptide, resulting in such peptido- and organomimetics of the peptides having substantial specific inhibitory activity. For computer modeling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press: Buffalo Grove, IL, pp. 165-174 and Principles of Pharmacology (ed. Munson, 1995), chapter 102 for a description of techniques used in computer assisted drug design. Example 22 describes other methods which can be used to generate mimetics.

Mutant VIAF gene: A mutant form of a VIAF gene which in some embodiments is associated with disease, for example: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; B-cell acute lymphoblastic leukemia; diabetes mellitus, transient neonatal diabetes; and insulin dependent diabetes.

Mutant VIAF RNA: The RNA transcribed from a mutant VIAF gene.

Mutant VIAF protein: The protein encoded by a mutant VIAF gene.

Neoplasm: Abnormal growth of cells.

Normal cells: Non-tumor, non-malignant cells.

Olig nucleotide: A linear polynucleotide sequence of up to about 200 nucleotide bases in length, for example a polynucleotide (such as DNA or RNA) which is at least 6 nucleotides, for example at least 15, 25, 50, 100 or even 200 nucleotides long.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

ORF (open reading frame): A series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are usually translatable into protein.

Ortholog: Two nucleotide sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

PCR (polymerase chain reaction): Describes a technique in which cycles of denaturation, annealing with primer, and then extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful herein are conventional. *Remington's Pharmaceutical Sciences*, by Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the DNA, RNA, proteins, and antibodies herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol, ethanol, combinations thereof, or the like, as a vehicle. The carrier and composition can be sterile, and the formulation suits the mode of administration. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, sodium saccharine, cellulose, magnesium carbonate, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

Probes and primers: Nucleic acid probes and primers may readily be prepared based on the amino acid sequences provided herein. A probe comprises an isolated nucleic acid attached to a

detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989) and Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences (1987).

Primers are short nucleic acids, such as DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), Ausubel *et al.*, 1987, and Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, 1990, Innis *et al.* (eds.), 21-27, Academic Press, Inc., San Diego, California. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

Polynucleotide: A linear nucleic acid sequence of any length. Therefore, a polynucleotide includes molecules which are 15, 50, 100, 200 (oligonucleotides) and also nucleotides as long as a full length cDNA.

Promoter: An array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein is more enriched than the peptide or protein is in its natural environment within a cell. For example, a preparation is purified when the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

Sample: Includes biological samples containing genomic DNA, RNA, or protein obtained from the cells of a subject, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, amniocentesis samples and autopsy material.

Sequence identity: The similarity between two nucleic acid sequences, or two amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or orthologs of nucleic acid or amino acid sequences will possess a relatively high degree of sequence identity when aligned using standard methods. This homology will be more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (e.g., human and chimpanzee sequences), compared to species more distantly related (e.g., human and *C. elegans* sequences). Typically, VIAF orthologs are at least 50% identical at the nucleotide level and at least 50% identical at the amino acid level when comparing orthologus sequences.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet et al., *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al*,. *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

Homologs of the VIAF proteins are typically characterized by possession of at least 60% sequence identity counted over full-length alignment with the amino acid sequence of human VIAF using the NCBI Blast 2.0, gapped blastp set to default parameters. Queries searched with the blastn program are filtered with DUST (Hancock, and Armstrong, 1994, *Comput. Appl. Biosci.* 10:67-70). Other programs use SEG. Alternatively, one may manually align the sequences and count the number of identical amino acids. This number divided by the total number of amino acids in the reference sequence multiplied by 100 results in the percent identity.

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer

than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 70%, 75%, 80%, 90%, 95%, 98%, 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85%, 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site.

One of ordinary skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. Provided herein are the peptide homologs described above, as well as nucleic acid molecules that encode such homologs.

An alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence-dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5 C to 20 C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence remains hybridized to a perfectly matched probe or complementary strand. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook *et al.* ((1989) In *Molecular Cloning: A Laboratory Manual*, CSHL, New York) and Tijssen ((1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* Part I, Chapter 2, Elsevier, New York). Nucleic acid molecules that hybridize under stringent conditions to a VIAF gene sequence will typically hybridize to a probe based on either an entire VIAF gene or selected portions of the gene under wash conditions of 2x SSC at 50 C. A more detailed discussion of hybridization conditions is presented in EXAMPLE 13.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein.

Such homologous peptides may, for example, possess at least 75%, 80%, 90%, 95%, 98%, or 99% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs may, for example, possess at least 75%, 85% 90%, 95%, 98% or 99% sequence identity over short windows of 10-20 amino acids. Methods for determining sequence identity over such short windows can be found at the NCBI web site. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it

is entirely possible that strongly significant homologs or other variants could be obtained that fall outside of the ranges provided.

The disclosure provides not only the peptide homologs that are described above, but also nucleic acid molecules that encode such homologs.

An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

Specific binding agent: An agent that binds substantially only to a defined target. As used herein, the term "VIAF peptide specific binding agent" includes anti-VIAF peptide antibodies and other agents that bind substantially only to the VIAF peptide. The antibodies may be monoclonal or polyclonal antibodies that are specific for the VIAF peptide, as well as immunologically effective portions ("fragments") thereof. In one embodiment, the antibodies used herein are monoclonal antibodies (or immunologically effective portions thereof) and may also be humanized monoclonal antibodies (or immunologically effective portions thereof). Immunologically effective portions of monoclonal antibodies include Fab, Fab', $F(ab')_2$, Fabc and Fv portions (for a review, see Better and Horowitz, *Methods. Enzymol.* 178:476-96, 1989). Anti-inhibitory peptide antibodies may also be produced using standard procedures described in a number of texts, including *Antibodies, A Laboratory Manual* by Harlow and Lane, Cold Spring Harbor Laboratory (1988).

The determination that a particular agent binds substantially only to the VIAF peptide may readily be made by using or adapting routine procedures. One suitable *in vitro* assay makes use of the Western blotting procedure (described in many standard texts, including *Antibodies, A Laboratory Manual* by Harlow and Lane). Western blotting may be used to determine that a given VIAF peptide binding agent, such as an anti-VIAF peptide monoclonal antibody, binds substantially only to the VIAF protein.

Specifically hybridizable and specifically complementary: Terms which indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions in which specific binding is

desired, for example under physiological conditions in the case of *in vivo* assays. Such binding is referred to as "specific hybridization." See EXAMPLE 13 for hybridization conditions.

Subject: Living multicellular vertebrate organism, a category which includes, both human and veterinary subjects, for example, mammals, fish, and birds.

Sufficient complementarity: When used, indicates that a sufficient number of base pairs exist between the oligonucleotide and the target sequence to achieve detectable binding, and disrupt expression of gene products (such as VIAF). When expressed or measured by percentage of base pairs formed, the percentage complementarity that fulfills this goal can range from as little as about 50% complementarity to full, (100%) complementary. In general, sufficient complementarity is at least about 50%. In one embodiment, sufficient complementarity is at least about 75% complementarity. In another embodiment, sufficient complementarity is about 90% or about 95% complementarity. In yet another embodiment, sufficient complementarity is about 98% or 100% complementarity.

A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz *et al. Methods Enzymol* 100:266-285, 1983, and by Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Target sequence: A portion of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) or RNA that upon hybridization to an therapeutically effective oligonucleotide or oligonucleotide analog results in the inhibition of VIAF expression. Either an antisense or a sense molecule can be used to target a portion of dsDNA, since both will interfere with the expression of that portion of the dsDNA. The antisense molecule can bind to the plus strand, and the sense molecule can bind to the minus strand. Thus, target sequences can be ssDNA, dsDNA, and RNA.

An oligonucleotide "binds" or "stably binds" to a target nucleic acid if a sufficient amount of the oligonucleotide forms base pairs or is hybridized to its target nucleic acid, to permit detection of that binding. Binding can be detected by either physical or functional properties of the target:oligonucleotide complex. Binding between a target and an oligonucleotide can be detected by any procedure known to one skilled in the art, including both functional and physical binding assays. Binding may be detected functionally by determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation and the like.

Physical methods of detecting the binding of complementary strands of DNA or RNA are well known in the art, and include such methods as DNase I or chemical footprinting, gel shift and affinity cleavage assays, Northern blotting, dot blotting and light absorption detection procedures. For example, one method that is widely used, because it is so simple and reliable, involves observing a change in light absorption of a solution containing an oligonucleotide (or an analog)

and a target nucleic acid at 220 to 300 nm as the temperature is slowly increased. If the oligonucleotide or analog has bound to its target, there is a sudden increase in absorption at a characteristic temperature as the oligonucleotide (or analog) and target disassociate or melt.

The binding between an oligomer and its target nucleic acid is frequently characterized by the temperature (T_m) at which 50% of the oligomer is melted from its target. A higher (T_m) means a stronger or more stable complex relative to a complex with a lower (T_m).

Therapeutically Effective Amount: A concentration of VIAF, for example an amount that is effecitve to modulate (increase or decrease) the anti-apoptotic and signaling properties of the IAP family in a subject to whom it is administered. In particular detailed examples, it is an amount required to inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example more than about 1.5 fold, for example more than about 2-fold. In other examples, it is an amount required when co-expressed with suboptimal quantities of XIAP, will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli. In yet other examples, it is an amount required to synergistically stimulate c-Jun N-terminal kinase activity. Such inhibition will decrease (including preventing) apoptosis in a cell, such as the cell of a patient.

The therapeutically effective amount also includes a quantity of VIAF protein sufficient to achieve a desired effect in a subject being treated. For instance, this can be the amount necessary to improve signs and/or symptoms a disease of abnormal apoptosis, for example by modulating the anti-apoptotic and signaling properties of the IAP family.

An effective amount of VIAF may be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount of VIAF will be dependent on the source of VIAF applied (i.e. VIAF isolated from a cellular extract versus a chemically synthesized and purified VIAF, or a variant or fragment that may not retain full VIAF activity), the subject being treated, the severity and type of the condition being treated, and the manner of administration of VIAF. For example, a therapeutically effective amount of VIAF can vary from about 0.01 mg/kg body weight to about 1 g/kg body weight.

The VIAF protein disclosed herein has equal application in medical and veterinary settings. Therefore, the general term "subject being treated" is understood to include all animals (e.g. humans, apes, dogs, cats, horses, and cows) that require modulation of the anti-apoptotic and signaling properties of the IAP family activity that is susceptible to VIAF-mediated modulation.

Therapeutically effective dose: A dose sufficient to modulate the apoptotic and signaling properties of the IAP family, resulting in a regression of a pathological condition, or which is capable of relieving signs or symptoms caused by the condition, such as cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; B-cell acute lymphoblastic leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Transgenic Cell: Transformed cells which contain foreign, non-native DNA.

Tumor: A neoplasm

Variants or fragments or fusion proteins: The production of VIAF proteins can be accomplished in a variety of ways (for example see EXAMPLES 6, 13, 14 amd 29). DNA sequences which encode for the protein or fusion protein, or a fragment or variant of the protein, can be engineered such that they allow the protein to be expressed in eukaryotic cells, bacteria, insects, and/or plants. In order to accomplish this expression, the DNA sequence can be altered and operably linked to other regulatory sequences. The final product, which contains the regulatory sequences and the therapeutic protein, is referred to as a vector. This vector can then be introduced into the eukaryotic cells, bacteria, insect, and/or plant. Once inside the cell the vector allows the protein to be produced.

One of ordinary skill in the art will appreciate that the DNA can be altered in numerous ways without affecting the biological activity of the encoded protein. For example, PCR may be used to produce variations in the DNA sequence which encodes VIAF. Such variants may be variants that are optimized for codon preference in a host cell that is to be used to express the protein, or other sequence changes that facilitate expression.

Two types of cDNA sequence variant may be produced. In the first type, the variation in the cDNA sequence is not manifested as a change in the amino acid sequence of the encoded polypeptide. These silent variations are simply a reflection of the degeneracy of the genetic code. In the second type, the cDNA sequence variation does result in a change in the amino acid sequence of the encoded protein. In such cases, the variant cDNA sequence produces a variant polypeptide sequence. In order to optimize preservation of the functional and immunologic identity of the encoded polypeptide, any such amino acid substitutions may be conservative. Conservative substitutions replace one amino acid with another amino acid that is similar in size, hydrophobicity, etc.

Variations in the cDNA sequence that result in amino acid changes, whether conservative or not, are minimized to enhance preservation of the functional and immunologic identity of the encoded protein. The immunologic identity of the protein may be assessed by determining whether it is recognized by an antibody to VIAF; a variant that is recognized by such an antibody is immunologically conserved. In particular embodiments, any cDNA sequence variant will introduce no more than 20, for example fewer than 10 amino acid substitutions into the encoded polypeptide. Variant amino acid sequences can, for example, be 80%, 90% or even 95% identical to the native

amino acid sequence. In other embodiments, the VIAF protein has less than 275 amino acids, for example less than 250, 240, or 237 amino acids. Alternatively, the VIAF protein may have 237, 236, 200, 150, or 100 amino acids.

Conserved residues in the same or similar proteins from different species can also provide guidance about possible locations for making substitutions in the sequence. A residue which is highly conserved across several species is more likely to be important to the function of the protein than a residue that is less conserved across several species.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

VIAF Biological Activity: Such biological activity includes the ability to modulate (and especially increase) the anti-apoptotic and signaling properties of the IAP family. Alternatively (or in addition), the protein has the ability to inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example by a factor of at least 1.5, for example at least 2, (where the factor refers to at least approximately double the cell viability using the assay described in EXAMPLE 9). Alternatively (or in addition), the protein has the ability to bind, interact with OpIAP, XIAP, c-IAP1 and c-IAP2 as determined by the assays described in EXAMPLES 7 and 8. In other embodiments, co-expression of the protein with suboptimal quantities of XIAP will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli (where the factor refers to the increase in cell viability using the assay described in EXAMPLE 9). In yet another embodiment, the protein can synergistically stimulate c-Jun N-terminal kinase activity using the methods described in EXAMPLE 10. In very particular embodiments, the biological activity includes any combination of the characteristics in this paragraph, or all of them.

VIAF cDNA: The *VIAF* cDNA is functionally defined as a cDNA molecule which encodes a protein having VIAF biological activity. The *VIAF* cDNA is derived by reverse transcription from the mRNA encoded by the *VIAF* gene and lacks internal non-coding segments and transcription regulatory sequences found in the *VIAF* gene.

VIAF fusion protein: A fusion protein comprising a VIAF protein (or variants, mutants, or fragments thereof) linked to other amino acid sequences.

VIAF gene: A gene which encodes a protein having VIAF biological activity. The definition of an VIAF gene includes the various sequence polymorphisms that may exist in other species.

VIAF protein: The protein encoded by VIAF cDNA. This protein may be functionally characterized by its biological ability as described above. VIAF proteins include the full-length cDNA transcript (SEQ ID NOs 2, 4, 6, 8 and 10), as well as shorter peptides which retain VIAF biological activity.

ZVAD-fmk: Tripeptide derivativebenzyloxycarbonyl-Val-Ala-Asp (Ome)-fluoromethyl ketone. A highly specific, cell-permeable and irreversible inhibitor of caspase-1- like proteases. Inhibits Fas-mediated apoptosis in Jurkat cells.

Additional definitions of common terms in molecular biology may be found in Lewin, B. *Genes V* published by Oxford University Press.

EXAMPLE 1

Cloning of a Human VIAF cDNA

A human VIAF was cloned using the yeast two-hybrid system. Yeast two-hybrid library screening and analysis were performed as described in the Matchmaker protocol provided by Clontech (Palo Alto, CA) using the yeast strain Y153. OpIAP (baculoviral IAP) was used as a bait to perform a yeast two-hybrid screen of a B cell lymphoma library. The known interaction between CD30 and TRAF2 was used as a positive control.

Competent Y153 yeast cells were transformed with the appropriate pAS1 and pACT plasmid DNA derivatives (for library screening 30 μg of each plasmid) containing fish sperm carrier DNA. To transform the yeast cells, the DNA and yeast were combined with polyethylene glycol/LiOAc and incubated one hour at 30°C, then for 25 minutes at 42°C. The yeast cells were then plated on plates lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole (3-AT) and plates lacking leucine and tryptophan. After three days at 30°C, colonies from plates lacking leucine, tryptophan, and histidine and containing 3-AT were transferred to filters and assayed for β-galactosidase activity. In all cases, colonies from the plates lacking leucine and tryptophan gave identical results. In general, β-galctosidase activity was apparent within one hour, but the filters were allowed to incubate for 24 hours. In all assays, transformants were tested for both the ability to grow on medium lacing histidine and containing 3-AT and for β-galactosidase expression.

Three overlapping, independent groups of clones, whose in-frame coding sequences possessed identical C-termini, were isolated. These clones interacted specifically with OpIAP but not a variety of negative controls (Table 1), including the GAL4 DNA binding domain alone (Durfee *et al.*, 1993, *Genes Dev.* 7:555-69), the cytoplasmic domain of the TNF receptor family member CD30 (Gedrich *et al.*, 1996, *J. Biol. Chem.* 271:12852-8), and the yeast SNF1-associated protein SNF4 (Durfee *et al.*, 1993, *Genes Dev.* 7:555-69). The OpIAP-interacting clones also scored negative against the homologous AcIAP, a non-functional IAP from *Autographa californica* nuclear polyhedrosis virus (Table 1), indicating that the ability of OpIAP to interact with this protein might be an important determinant of anti-apoptotic function.

Table 1: VIAF Interacts with OpIAP but not AcIAP

		Prey		
		Vector	VIAF	TRAF2
Bait	Vector	-	-	-
	CD30	-	-	+++
	OpIAP	-	+++	-
	AcIAP	-	-	-

The sequence obtained from the yeast two-hybrid screen was used to perform an EST database search. The full-length EST cDNA was subcloned into pBlueScript SK(+) (Stratagene), using EcoRI and NotI as cloning sites. All cDNA clones were fully sequenced in both directions. The resulting cDNA sequence (SEQ ID NO 1) encodes a novel 239 amino acid protein (SEQ ID NO 2) with a predicted molecular weight of 28 kDa (FIG. 1) which was designated VIAF (viral IAP-associated factor). The smallest VIAF clone obtained through two-hybrid screening contained the C-terminal 128 residues (amino acids 112 to 239 of SEQ ID NO 2) demonstrating this region is necessary and sufficient for interaction with OpIAP.

The VIAF open reading frame exhibited no obvious homology to caspases, IAPs or other proteins currently known to be involved in the regulation or execution of the apoptotic pathway. VIAF was has limited homology (27% over 165 residues) to phosducin, a cytosolic protein that interacts with the βγ subunits of G proteins. Immunofluorescence studies using 293 cells transfected with epitope-tagged VIAF demonstrated that VIAF is exclusively cytoplasmic.

Human VIAF maps to chromosome location 6q23.1 to 6q24.3. Several diseases associated with this region include: heterocellular hereditary persistence of fetal hemoglobin (142470); deafness (601316); cardiomyopathy (602067); B-cell acute lymphoblastic leukemia (designated as the six-twelve leukemia gene; 602532); diabetes mellitus, transient neonatal diabetes (601410) and insulin dependent diabetes (606320). Numbers in parenthesis correspond to the Online Mendelian Inheritance in Man Gene Map numbers. Additional information can be found on the Internet through the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894), for example the website of the Online Mendelian Inheritance in Man Gene Map. These are example of diseases associated with abnormal apoptosis. Leukemia, for example, is a malignancy in which malignant cells do not undergo normal apoptosis. Cardiomyopathy and diabetes are examples of conditions in which cells (such as myocardial cells or pancreatic islet cells) undergo unwanted apoptosis.

EXAMPLE 2

Cloning of VIAF in Other Organisms

A VIAF gene was cloned in several different organisms including: mouse, zebrafish, *Drosophila* and yeast (*S. cerevisiae*). Specific primers were designed to perform PCR to obtain the VIAF homologues.

The following primers were used to clone VIAF in several species: human VIAF sense primer: ATAGGATCCATGGAGGACCCCAACGCAGACACTG (SEQ ID NO 11); human antisense primer: 5'-AATATCGATCCAGACAATTTGTCACAAGAAAGTTCG-3' (SEQ ID NO 12); mouse VIAF sense primer: 5'-AATGGATCCATGCAGGACCCCAATGCAGACACC-3' (SEQ ID NO 13); mouse antisense primer: 5'- ATTATCGATTCAAAGGTTCCATCACTGCCA-3' (SEQ ID NO 14); zebrafish sense primer: 5'-ATAGGATCCATGCAGGACCCAAAC GACACCGAGTGGAAC-3' (SEQ ID NO 15); zebrafish antisense-primer: 5'-AATATCGATCGTGGGCAGGTTGCGGTCGGGGTAGTTGGG -3' (SEQ ID NO 16); *Drosophila* VIAF: sense primer: 5'-TAAATCGATATGCAGGACCCAAACGAAGATACC-3' (SEQ ID NO 17); *Drosophila* antisense primer: 5'-ATAATCGATTGCCGGTTTTGGATTGGG-3' (SEQ ID NO 18); yeast VIAF sense primer: 5'-AATGGATCCATGGAGAATGAACCAATGTTTCAGG-3' (SEQ ID NO 19); and yeast antisense primer: 5'-ATAGCGGCCGCCTGTAAATAAGGAATATTGGCA-3' (SEQ ID NO 20).

For all of the VIAF cloning experiments, the following PCR conditions were used: 94°C for 5 minutes; 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 2 minutes (30 cycles); 72°C for 10 minutes. The mouse and yeast homologues were amplified using DNA from a mouse T cell library and yeast genomic DNA, respectively. The *Drosophila* library used was the Matchmaker 2hybrid library, (Clontech, Cat # IL 4002AH), and the missing 3'-end was amplified with Marathon cDNA Amplification Kit, (Clontech Cat # K1802-1). The zebrafish cDNA was cloned using EST clone #fb37bO3 (Research Genetics, AL).

VIAF cDNA was cloned in several species, and the protein encoded by the cDNA determined: mouse (SEQ ID NOS 3 and 4), zebrafish (SEQ ID NOS 5 and 6), *Drosophila* (SEQ ID NOS 7 and 8), and yeast (SEQ ID NOS 9 and 10) were cloned. The approximate percent identity to the human VIAF nucleic acid sequence (SEQ ID NO 1) to the mouse, zebrafish, *Drosophila* and yeast VIAF nucleic acid sequences is 84.9%, 70.8%, 59.0%, and 48%, respectively.

As shown in FIG. 1, there is a high degree of conservation of the VIAF amino acid sequence between different species. The average percent identity to the human clone between the mouse, zebrafish, *Drosophila* and yeast homologues at the amino acid level is 90%, 74.8%, 51.8% and 32.3% similar, respectively.

EXAMPLE 3

Structure of VIAF

The structure of VIAF predicted from the amino acid sequences of the proteins obtained in EXAMPLES 1 and 2 is shown in FIG. 2. There is a predicted coiled coil domain (amino acids 23-89), predicted SHC SH2 domain binding site (amino acids 48-52), two predicted ATP binding sites (amino acids 83-96 and 100-111) and a predicted OpIAP interaction domain (amino acids 111-239). The amino acid numbers refer to those shown in SEQ ID NO 2.

Additional studies were conducted to determine if VIAF is a phosphoprotein. Human embryonic kidney cells (293 cells) were transfected with 2 μg of full-length human VIAF (SEQ ID NO 1) cloned into the expression vector pEBB (Mayer *et al.*, 1995, *Curr.Biol.* 5:296-305), or the vector alone. Six hours after transfection, cells were incubated for 12 hours in phosphate-free RPMI 1640 and then labeled for 24 hours with [^{32}P]-orthophosphate. Radioactive media was removed, cells were washed five times with serum-free RPMI 1640 and then lysed with Triton-X-100 containing buffer. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and labeled proteins were visualized by autoradiography. The results demonstrated that VIAF is a phosphoprotein.

EXAMPLE 4

Northern Analysis of VIAF RNA Expression

VIAF mRNA expression was analyzed using Northern blot analyses. Full-length human VIAF cDNA (SEQ ID NO 1) was labeled with [α^{32}P]dATP using the Rediprime II kit (Amersham Pharmacia Biotech, Piscataway, NJ, Cat # RPN 1633) as instructed by the manufacturer. Briefly, the cDNA (100 ng) was combined with α-[^{32}P] dATP (4 μl) and the mixture incubated for 30 minutes at 37°C. Unincorporated radiolabel was separated from the labeled cDNA by centrifuging it through a G50-Sephadex column. The radiolabeled human VIAF cDNA was used to probe a multiple adult human tissue blot (Clontech). Hybridization was carried out under high stringency conditions according to the manufacturers instructions. The blot was prehybridized for 30 minutes at 68°C with ExpressHyb solution (Clontech), and then hybridized for one hour at 68°C in ExpressHyb. The blot was washed four times at room temperature (RT) with 2x SSC, 0.1% SDS and two times at 50°C with 0.1x SSC, 0.1% SDS. The blot was exposed overnight at -80°C with intensifier screens. As a control, the blot was incubated with a radioactive probe for β-actin. The cDNA for β-actin (Clontech) was radiolabeled as described above using the Rediprime II kit.

As shown in FIG. 3, Northern blot analyses revealed a major band of approximately 1.2 kb with the VIAF probe, and a major band of 2.0 kb with the β-actin control probe. VIAF mRNA is ubiquitously expressed but its mRNA expression is most abundant in the testis, followed by ovary and prostate. Expression of VIAF is minimal in the thymus.

EXAMPLE 5

Generation of Polyclonal Antibodies Against VIAF

The example describes a method used to generate polyclonal antibodies against human VIAF. Similar experiments can be used to produce polyclonal antibodies to VIAF from other species, such as zebrafish VIAF, using the protein sequences disclosed herein. In addition, similar experiments can be used to generate polyclonal antibodies that recognize other VIAF fragments, variants, or mutant forms thereof.

Rabbit polyclonal sera was raised against keyhole limpet hemocyanin (KLH)-conjugated peptides encoding the N- and C-termini of VIAF. The peptide sequence for the antibody against the N-terminal end was: (KLH)-MQDPNADTEWNDILR (SEQ ID NO 21) and the sequence for the C-terminal end was: (KLH)-RRSVLMKRDSDSEGD (SEQ ID NO 22).

The antibody titer was determined by ELISA assay with free peptide bound in solid phase (1 μg/well). Results were expressed as the reciprocal of the serum dilution that results in an OD_{405} of 0.2. Detection was obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

To demonstrate that the antibodies specifically recognized the VIAF protein, the antibodies were used to probe a membrane containing recombinant VIAF. Recombinant VIAF proteins (full-length cDNAs, SEQ ID NOs 1, 3, 5, and 9) were each cloned into a pEBB expression vector (Mayer *et al.*, 1995, *Curr.Biol.* 5:296-305). Expression of the VIAF cDNA using this vector results in a VIAF fusion protein containing a Flag-tag. The peBB-VIAF encoding vectors were transfected into 293 cells. After allowing expression of the recombinant VIAF, the cells were lysed (1% Triton-X-100, 1 mM EDTA, 100 mM NaCl, 50mM Tris-HCl [pH 8.0]) and the proteins resolved using SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, which was probed with a mixture of the polyclonal antibodies generated above. The membrane was subsequently incubated with the secondary antibody donkey anti-rabbit Ig, horseradish peroxidase-conjugated linked whole antibody (Amersham Pharmacia Biotech Cat # NA934). The VIAF proteins were visualized using Enhanced Chemiluminescence.

The VIAF polyclonal antibodies recognized recombinant Flag-tagged VIAF proteins (approximately 30 kDa) of all species tested (human, mouse, *Drosophila*) except yeast. As a negative control, samples containing only the pEBB-Flag vector alone were used. Since the polyclonal antibodies did not recognize the Flag-tag, this demonstrates that the antibodies specifically recognize the VIAF protein. In addition, this demonstrates that the cloned cDNAs encode native VIAF proteins.

VIAF-specific antibodies can be generated using the above method, or by using methods described in EXAMPLE 15.

EXAMPLE 6

Expression of Recombinant GST-VIAF

This example describes the expression of recombinant GST-VIAF in *E. coli*. Full-length human VIAF cDNA (SEQ ID NO 1) was cloned into a pGEX-2TK vector (Amersham Pharmacia Biotech) using the SmaI and EcoRI cloning sites. Expression of the VIAF cDNA using this vector results in a VIAF fusion protein containing a GST (glutathione-S-transferase) tag.

The plasmid was transformed into XL 1-Blue *E. coli* (Stratagene, La Jolla, CA). Bacteria were grown in 500 ml LB media until an OD_{600} of 0.6 was achieved. Cells were subsequently induced with 1 mM IPTG for three hours. The bacteria cells were then lysed and sonicated in 50 ml GST-lysis buffer (1% Triton-X-100, 1 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl [pH 8.0]). The resulting lysates were centrifuged at 6000 rpm for 30 minutes and supernatant incubated for two hours with 500 μl of a 50% slurry of Protein G beads (Amersham Pharmacia Biotech). The beads were washed five times with lysis buffer. These beads were used directly for the precipitation experiments described below.

Aliquots containing 10 μl beads were loaded onto a SDS-polyacrylamide gel and stained with Colloidal Blue Staining Kit (NOVEX, San Diego, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The VIAF proteins were detected using a mixture of the polyclonal antibodies generated in EXAMPLE 5. The blot was subsequently incubated with the secondary antibody donkey anti-rabbit Ig, horseradish peroxidase-conjugated linked whole antibody (Amersham Pharmacia Biotech, Cat # NA934). The gels were fluorographed with Enlightning solution (NEN, Boston, MA) and VIAF proteins visualized by autoradiography.

EXAMPLE 7

VIAF Interacts with OpIAP

This example describes a precipitation assay used to demonstrate that VIAF interacts with OpIAP *in vitro*. OpIAP (0.5 μg plasmid DNA) was *in vitro* translated from the Bluescript plasmid templates with ^{35}S-labeled methionine in rabbit reticulolysates (30 μCi/sample) using the TNT T7/T3-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions.

For GST precipitation experiments, VIAF was subcloned into pGEX-2TK (Amersham Pharmacia Biotech). GST-VIAF fusion protein and GST control protein were expressed in XL-1 *E. coli* (Stratagene, La Jolla, CA) and purified as described previously (Gedrich *et al.*, 1996, *J. Biol. Chem.* 271:12852-8, herein incorporated by reference). Coprecipitation experiments with *in vitro* translated proteins were performed as described previously (*Id.*), except that Triton buffer was used for washes (25 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10%

glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride and cocktail of protease inhibitors). The precipitated proteins were separated by SDS-PAGE. Gels were fluorographed with Enlightning solution (NEN, Boston, MA) and visualized by autoradiography.

As shown in FIG. 4, recombinant GST-VIAF, but not GST alone, associates with OpIAP *in vitro*. The input lanes are the total cell lysates, prior to precipitation, showing that the OpIAP proteins were *in vitro* translated.

EXAMPLE 8

Identification of IAPs which Interact with VIAF

As shown in EXAMPLE 7, VIAF interacts with baculoviral OpIAP *in vitro*. To determine if VIAF interacts with human IAP proteins, co-immunoprecipitation experiments were conducted.

The mammalian IAP XIAP (ILP-1) functions as a downstream inhibitor of cell death through its association and enzymatic inhibition of Caspase-3. To determine whether endogenous human VIAF and XIAP proteins interact, the following experiment was performed. Four semi-confluent 10 cm dishes (approximately 1.5 x 10^7 cells per plate) of 293 cells were washed twice with phosphate-buffered saline and then lysed for 10 minutes on ice in 500 μl per plate of Triton buffer supplemented with 1 mM sodium orthovanadate and 1 mM sodium fluoride. Lysates were pooled and incubated for two hours at 4°C together with a cocktail of VIAF polyclonal sera (see EXAMPLE 5) and precipitated for an additional hour with 50 μl of protein G agarose (Life Technologies, Rockville, MD). The precipitates were washed three times and immunoblotted with the anti-VIAF polyclonal antibodies or with an anti-XIAP monoclonal antibody (Transduction Laboratories, Lexington, KY). Blots were resolved using either horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies contained within the Enhanced Chemiluminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech). XIAP coimmunoprecipitated with VIAF polyclonal antibodies but not with preimmune sera, indicating that VIAF and XIAP normally co-associate in cells (FIG. 5).

To further examine the VIAF-XIAP interaction, GST-VIAF protein was tested in coprecipitation experiments using several ^{35}S-labeled deletion mutants of XIAP generated by *in vitro* translation and precipitated with GST-VIAF. A truncation mutant of XIAP encoding only the three N-terminal BIR (baculovirus IAP repeat) domains was efficiently precipitated with GST-VIAF, demonstrating that the BIRs are necessary and sufficient for interaction with VIAF (FIG. 6, 3 BIR). However, a deletion mutant lacking the three BIRs could also coprecipitate, although with much less efficiency (FIG. 6, Δ BIR), suggesting that more that one VIAF binding site may exist in XIAP. GST-VIAF did not coprecipitate Caspase-9, confirming the specificity of the VIAF-XIAP interaction.

To determine if human VIAF interacts with other mammalian IAP proteins, co-precipitation experiments were performed with GST-VIAF and *in vitro* translated ^{35}S-labeled XIAP, c-IAP1 and c-IAP2 proteins. These IAP proteins were efficiently precipitated by GST-VIAF (FIG. 7), but not by GST alone, indicating that VIAF can also associate with c-IAP1 and c-IAP2.

EXAMPLE 9

VIAF Regulates Bax- and Fas-Mediated Apoptosis

To determine whether VIAF could regulate apoptosis, an expression vector encoding VIAF was tested for its ability to regulate apoptosis induced by Bax and Fas.

For Bax-induced apoptosis assays, 293 cells were transfected by calcium phosphate precipitation for 15 hours with 0.5 μg of a plasmid encoding β-galactosidase, 0 - 4 μg of a plasmid encoding human VIAF (see EXAMPLE 3) together with: 2 μg pcDNA3 ( ), 1 μg Bax (■), 1 μg Bax and 0.1 μg XIAP (▼), or 1 μg Bax and 0.5 μg XIAP (●) (FIG. 8A). For Fas-induced apoptosis assays, 293 cells were transfected by calcium phosphate precipitation for 20 hours with 0.5 μg of a plasmid encoding β-galactosidase, 0 - 4 μg of a plasmid encoding human VIAF (see EXAMPLE 3) together with: 2 μg of pcDNA3 ( ), 2 μg of Fas (■), 2 μg of Fas and 0.1 μg XIAP (▼) or 2 μg of Fas and 0.5 μg of XIAP (●). The total amount of DNA was equalized with control vector. Cells death of transfected cells was counted by morphology. Viable cells are spread out and adherent to the cell culture dish. In contrast, apoptotic cells detach from the culture dish and have a rounded shape. In addition, the cell membrane of apoptotic cells is blebed and in later stages of apoptosis the cell dissolves into apoptotic bodies.

Transfection of VIAF into 293 cells inhibited Bax-mediated apoptosis in a dose-responsive manner (FIG. 8A), although this protection reached a plateau at two micrograms of transfected plasmid, indicating that VIAF exerts its effects through a cellular factor whose concentration is limiting. Coexpression of XIAP at levels which alone only partially protect against Bax were almost completely protective when coexpressed with VIAF (FIG. 8A). Similarly, VIAF enhanced the ability of XIAP to protect from Fas-mediated apoptosis (FIG. 8B). Almost identical results were obtained using VIAF and OpIAP. These results demonstrate that VIAF can substantially protect cells from both Fas- and Bax-induced apoptosis, and co-expression of VIAF with suboptimal quantities of XIAP confers almost complete protection from these stimuli.

EXAMPLE 10

VIAF and XIAP have a Synergistic Effect on JNK activation

In addition to their caspase-inhibitory properties, IAPs are also involved in the regulation of c-Jun N-terminal kinase (JNK). JNK is required for the protective role of XIAP (Sanna *et al.* 1998, *Proc. Natl. Acad. Sci. USA* 95:6015-20). To further investigate the mechanism of VIAF, the role of VIAF in these functions was examined.

Kinase assays were performed as follows. Cell lysate (100 μl) was incubated for one hour at 4°C on rotator with one μl of HA antibody (12CA5 Boehringer Mannheim) and 10 μl of protein A beads (Pierce) which were previously washed and resuspended in 100 μl of M2 buffer (20 mM Tris, pH 7.6; 0.5% NP40; 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.2 mM DTT, 0.05 mM PMSF, 20 mM beta-glycerophosphate, 0.1 mM sodium vandate). The beads were washed twice with M2 buffer followed by two washings with kinase buffer (20 mM Hepes, pH 7.5; 20 mM beta-glycerophosphate, 10 mM PNPP, 10 mM $MgCl_2$, 1 mM DTT, 50 μM sodium vanadate). The beads were incubated for 30 minutes in 50 μl kinase buffer containing 20 μM ATP and 0.5 μl gamma-ATP (0.5 μCi) and 2 μg GST-cjun. Samples were subjected to SDS-page and the gel dried and exposed to autoradiography. Activation of JNK was detected by phosphorylation of GST-c-jun (1-79) (FIG. 9, c-jun-P). Expression of VIAF and XIAP was confirmed by immunoblotting of lysates with antibodies to VIAF and XIAP as described in EXAMPLE 8. Quantitation of JNK activity was performed by phosphorimage analysis using Storm 840 and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

As shown in FIG. 9, coexpression of VIAF and XIAP synergistically induced JNK activation, indicating that that VIAF may exert its effects by potentiating the JNK-inducing properties of XIAP.

While both Fas and Bax induce the activation of Caspase-3, in 293 cells this is achieved through two distinct pathways (Hakem *et al.*, 1998, *Cell* 94:339-52; Kuida *et al.*, 1998, *Cell* 94:325-37; Varfolomeev *et al.*, 1998, *Immunity* 9:267-76). Fas-induced death occurs in a manner which does not require activation of Caspase-9 and which is not blocked by dominant negative Caspase-9, while Bax-induced death occurs through the activation of a Caspase-9:Apaf1 holoenzyme complex, and therefore is blocked by expression of dominant negative Caspase-9. Taken together, these data indicate that the point of action of VIAF is downstream in the apoptotic cascade after the Fas- and Bax-induced pathways have converged, and that VIAF may function to enhance the pro-survival properties of XIAP or of other endogenous IAP proteins.

EXAMPLE 11

Characterization of Endogenous VIAF in Murine Thymocytes

This example describes experiments conducted to determine the role of endogenous cellular VIAF in apoptosis. The cells used in this example were freshly isolated, not from a cell line. Similar experiments can be used to examine VIAF expression in other samples, such as biopsies or others.

Isolation of Murine Thymocytes

Mice (B6 black mice no older than three months in age) were sacrificed and sterilized with ethanol. The thymus was removed and placed in RPMI media containing 10% FCS. After

transferring the thymus into a new petri dish to eliminate the blood cells, the thymus was cut into small pieces and filtered through nylon mesh into a sterile FALCON tube. To the tube, 30 ml of RPMI media was added. The cells were counted and seeded into 6-well tissue culture plates containing 3-4 ml/well, 5 x 10^6 cells/ml. Murine thymocytes are not viable after 48 hours.

Induction of VIAF Expression with Dexamethasone

To determine if VIAF is endogenously expressed in primary cells and tissues, such that the VIAF does not have to be over-expressed to study VIAF function, the level of VIAF expression was examined in thymocytes. Murine thymocytes were isolated as described above and stimulated with dexamethasone (10^{-7} M) or control media for the indicated amount of time. Dexamethasone is a glucocorticoid which induces apoptosis in human and murine thymocytes. The cells were subsequently lysed with RIPA-lysis buffer (Tris-HCl 50 mM, 150 mM, 1% NP40, 0.1% SDS, and 0.5.% deoxycholate) and proteins separated by SDS-PAGE and transferred onto a nitrocellulose membrane. VIAF was detected with a mixture of the two polyclonal anti-VIAF antibodies described in EXAMPLE 5.

Endogenous VIAF expression increased as a result of stimulating the thymocytes with dexamethasone after four hours (FIG. 10, compare lanes 1 and 2). Therefore, thymocytes contain sufficient levels of endogenous VIAF to study its role in apoptosis. These results indicate that VIAF may be necessary to induce and/or regulate this apoptotic process.

Apoptosis Inhibitors

To further investigate the role of VIAF in the apoptosis of murine thymocytes, the effect of several inhibitors of apoptosis was investigated.

Murine thymocytes were stimulated with or without dexamethasone (as described above) and co-incubated with the inhibitors (50 μM ZVAD-fmk; 50 μM lactacystin; 50 μM calpain inhibitor I; or media) as shown in FIG. 10. Cells were subsequently lysed with RIPA-lysis buffer (see above) and proteins separated by SDS-PAGE and transferred onto a nitrocellulose membrane. VIAF was detected with a mixture of the two polyclonal anti-VIAF antibodies described in EXAMPLE 5.

As shown in FIG. 10, expression of VIAF was induced by dexamethasone and this induction can be inhibited by ZVAD-fmk. However, VIAF expression was not inhibited by either lactacystin or calpain inhibitor I.

EXAMPLE 12

Cloning VIAF in Other Organisms

Having presented the nucleotide sequences of several homologs of VIAF cDNA and the amino acid sequences of the encoded proteins, this disclosure now also facilitates the identification

of DNA molecules, and thereby proteins, which are the VIAF homologs in the same or other species, for example polymorphisms in the same species or homologs in other species, such other primates. These other homologs can be derived from those sequences disclosed, but which vary in their precise nucleotide or amino acid sequence from those disclosed. Such variants may be obtained through a combination of standard molecular biology laboratory techniques and the nucleotide and amino acid sequence information disclosed herein.

The VIAF homologs in other organisms may be identified by using the VIAF sequences to design probes, for example an oligonucleotide or polynucleotide. Such probes can be used to screen a genomic or cDNA library from any organism using standard hybridization methods. In addition, primers or degenerate primers covering regions of VIAF thought to be important for its function (for example the C-terminal 128 amino acids of SEQ ID NO 2), can be designed for use in a PCR reaction to amplify VIAF homologs from a genomic or cDNA library.

EXAMPLE 13

Production of Sequence Variants of VIAF cDNAs and Proteins

SEQ ID NOs 1, 3, 5, and 7 show the nucleotide sequences of several VIAF homolog cDNAs, and the amino acid sequences of the VIAF proteins encoded by these cDNAs are shown in SEQ ID NOs 2, 4, 6, and 8, respectively. The distinctive functional characteristic of VIAF is its ability to modulate the anti-apoptotic and signaling properties of the IAP family. This activity of the VIAF protein may readily be determined using the assays described above, for example those described in EXAMPLES 7-10.

Having presented the nucleotide sequence of several VIAF cDNAs and the amino acid sequence of these proteins, this disclosure facilitates the creation of DNA molecules, and thereby proteins, which are derived from those disclosed but which vary in their precise nucleotide or amino acid sequence from those disclosed. Such variants may be obtained through a combination of standard molecular biology laboratory techniques and the nucleotide sequence information disclosed herein.

VIAF variants and fragments will retain the ability to modulate the anti-apoptotic and signaling properties of the IAP family. Since the region containing amino acids 7-21 of the human VIAF sequence is highly conserved between species (see FIG. 1), in particular embodiments these residues of VIAF ideally do not substantially diverge from the wild-type sequence shown in SEQ ID NOs 2, 4, 6, and 8. In other embodiments, the VIAF protein has less than 275 amino acids, for example less than 250, 240, or 237 amino acids. Alternatively, the VIAF protein may have 237, 236, 200, 150, or 100 amino acids. Other important residues include those domains noted in FIG. 2. Such domains include, but are not limited to: the SHC SH2 domain binding site, amino acids 48-52 of SEQ ID NO 2, ATP binding sites, amino acids 83-96 and 100-111 of SEQ ID NO 2, and

the OpIAP interaction domain, amino acids 111-239 of SEQ ID NO 2. In these regions, conservative substitutions will be better tolerated than non-conservative substitutions.

The indication of highly conserved regions in FIG. 1 provides further guidance in helping select residues that may be substituted or deleted. For example, referring to FIG. 1, the region containing amino acids 33-40 of the human VIAF sequence is not highly conserved among the five species shown. Therefore, alterations in the sequence in this region are predicted to have less of an effect on the function of the VIAF protein, than for example mutations in the region containing amino acids 7-21 of the human VIAF sequence. Variants and fragments may retain at least 60%, 70%, 80%, 85%, 95%, 98%, or greater sequence identity to the VIAF amino acid sequences disclosed herein, and in particular embodiments at least this much identity to SEQ ID NOs 2, 4, 6, and 8. Less identity is allowed, as long as the variant VIAF sequence maintains the functional activity of the VIAF protein as defined herein. Such activity can be readily determined using the assays disclosed herein.

The simplest modifications involve the substitution of one or more amino acid residues (for example 2, 5 or 10 residues) for amino acid residues having similar biochemical properties. These so-called conservative substitutions are likely to have minimal impact on the activity of the resultant protein. Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are conservative when it is desired to finely modulate the characteristics of the protein. Examples of amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Ser for Cys; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val.

Amino acid substitutions are typically of single residues, for example 1, 2, 3, 4, 5, 10 or more substitutions; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. Obviously, the mutations that are made in the DNA encoding the protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those listed above, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in protein properties will be those in

which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine. Such variants can be readily selected for additional testing by performing an assay (such as that shown in EXAMPLE 9) to determine if the variant can modulate anti-apoptotic signaling, for example inhibit Bax- and Fas- induced apoptosis by more than a desired amount, for example more than about 1.5 fold, for example more than about 2-fold, and to determine if co-expression of the variant with suboptimal quantities of XIAP will confer almost complete protection, for example about 1.2 fold, for example 1.5 fold, from these stimuli. The ability of the variant to modulate the anti-apoptotic signaling properties of the IAP family can be readily assayed using the methods described in EXAMPLE 10. The ability of VIAF to interact with OpIAP, XIAP, c-IAP1 and c-IAP2 can also be readily assayed as in EXAMPLES 7 and 8.

The effects of these amino acid substitutions or deletions or additions may be assessed for derivatives of the VIAF protein by the assays as described in the EXAMPLES above.

Variant DNA molecules include those created by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Ch. 15). By the use of such techniques, variants may be created which differ in minor ways from those disclosed. DNA molecules and nucleotide sequences which are derivatives of those specifically disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein which possesses the functional characteristics of the VIAF proteins, are comprehended by this disclosure.

Also within the scope of this disclosure are small DNA molecules which are derived from the disclosed DNA molecules. Such small DNA molecules include oligonucleotides suitable for use as hybridization probes or polymerase chain reaction (PCR) primers. As such, these small DNA molecules will comprise at least a segment of the VIAF cDNA molecules or the VIAF gene and, for the purposes of PCR, will comprise at least a 20, 30, 40 or 50 contiguous nucleotides of the VIAF cDNAs or genes from SEQ ID NOs. 1, 3, 5, 7, 9 or their complementary strands. It will be appreciated that such longer length nucleotide sequences will provide greater specificity in hybridization or PCR applications than shorter length sequences. Accordingly, superior results may be obtained using these longer stretches of consecutive nucleotides.

DNA molecules and nucleotide sequences which are derived from the disclosed DNA molecules as described above may also be defined as DNA sequences which hybridize under stringent conditions to the DNA sequences disclosed, or fragments thereof. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the

hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Chapters 9 and 11), herein incorporated by reference.

Specific hybridization refers to the binding, duplexing, or hybridizing of a molecule only or substantially only to a particular nucleotide sequence when that sequence is present in a complex mixture (e.g. total cellular DNA or RNA). Specific hybridization may also occur under conditions of varying stringency.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989 ch. 9 and 11), herein incorporated by reference. By way of illustration only, a hybridization experiment may be performed by hybridization of a DNA molecule (for example, a variant of the VIAF cDNA) to a target DNA molecule (for example, the VIAF cDNA) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern, *J. Mol. Biol.* 98:503, 1975), a technique well known in the art and described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Hybridization with a target probe labeled with [^{32}P]-dCTP is generally carried out in a solution of high ionic strength such as 6xSSC at a temperature that is 20-25°C below the melting temperature, T_m, described below. For such Southern hybridization experiments where the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to 10^9 CPM/μg or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization. The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal.

The term T_m represents the temperature above which, under the prevailing ionic conditions, the radiolabeled probe molecule will not hybridize to its target DNA molecule. The T_m of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy, *Proc. Natl. Acad. Sci. USA* 48:1390, 1962): $T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - 0.63(\%\text{ formamide}) - (600/l)$; where l = the length of the hybrid in base pairs.

This equation is valid for concentrations of Na^+ in the range of 0.01 M to 0.4 M, and it is less accurate for calculations of T_m in solutions of higher [Na^+]. The equation is also primarily valid for DNAs whose G+C content is in the range of 30% to 75%, and it applies to hybrids greater than 100 nucleotides in length (the behavior of oligonucleotide probes is described in detail in Ch. 11 of Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Thus, by way of example, for a 150 base pair DNA probe derived from the open reading frame of the VIAF cDNA (with a hypothetical %GC = 45%), a calculation of hybridization conditions required to give particular stringencies may be made as follows: For this example, it is assumed that the filter will be washed in 0.3 xSSC solution following hybridization, thereby: [Na^+] = 0.045 M; %GC = 45%; Formamide concentration = 0; l = 150 base pairs; T_m=81.5 - 16.6($\log_{10}$[Na^+]) + (0.41 x 45) - (600/150); and so T_m = 74.4°C.

The T_m of double-stranded DNA decreases by 1-1.5°C with every 1% decrease in homology (Bonner et al., *J. Mol. Biol.* 81:123, 1973). Therefore, for this given example, washing the filter in 0.3 xSSC at 59.4-64.4°C will produce a stringency of hybridization equivalent to 90%; that is, DNA molecules with more than 10% sequence variation relative to the target VIAF cDNA will not hybridize. Alternatively, washing the hybridized filter in 0.3 xSSC at a temperature of 65.4-68.4°C will yield a hybridization stringency of 94%; that is, DNA molecules with more than 6% sequence variation relative to the target VIAF cDNA molecule will not hybridize. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

Examples of stringent conditions are those under which DNA molecules with more than 25%, 15%, 10%, 6% or 2% sequence variation (also termed "mismatch") will not hybridize. Stringent conditions are sequence dependent and are different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be no more than about 5°C lower than the thermal melting point T_m for the specific sequence at a defined ionic strength and pH. An example of stringent conditions is a salt concentration of at least about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and a temperature of at least about 30°C for short probes (e.g. 10 to 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For example, conditions of 5X SSPE (750 mM NaCl, 50 mM Na Phosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C are suitable for allele-specific probe hybridizations.

A perfectly matched probe has a sequence perfectly complementary to a particular target sequence. The test probe is typically perfectly complementary to a portion (subsequence) of the target sequence. The term "mismatch probe" refers to probes whose sequence is deliberately selected not to be perfectly complementary to a particular target sequence.

Transcription levels can be quantitated absolutely or relatively. Absolute quantitation can be accomplished by inclusion of known concentrations of one or more target nucleic acids (for example control nucleic acids such as Bio B or with a known amount the target nucleic acids themselves) and referencing the hybridization intensity of unknowns with the known target nucleic acids (for example by generation of a standard curve).

The degeneracy of the genetic code further widens the scope of the present disclosure as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. For example, the sixth amino acid residue of the human VIAF protein is alanine. This is encoded in the VIAF cDNA by the nucleotide codon triplet GCA. Because of the degeneracy of the genetic code, three other nucleotide codon triplets, GCT, GCG and GCC, also code for alanine. Thus, the nucleotide sequence of the VIAF cDNA could be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the cDNA molecules disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. DNA sequences which do not hybridize under stringent conditions to the cDNA sequences disclosed by virtue of sequence variation based on the degeneracy of the genetic code are herein also comprehended by this disclosure.

One skilled in the art will recognize that the DNA mutagenesis techniques described above may be used not only to produce variant DNA molecules, but will also facilitate the production of proteins which differ in certain structural aspects from the VIAF proteins, yet which proteins are clearly derivative of this protein and which maintain the essential characteristics of the VIAF protein. Newly derived proteins may also be selected in order to obtain variations on the characteristic of the VIAF protein, as described herein. Such derivatives include those with variations in amino acid sequence including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

The *VIAF* gene, *VIAF* cDNA, DNA molecules derived therefrom and the proteins encoded by these cDNAs and derivative DNA molecules may be utilized in aspects of both the study of VIAF and for diagnostic and therapeutic applications related to VIAF. Utilities disclosed herein include, but are not limited to, the modulation of anti-apoptotic and signaling properties of the IAP family. Those skilled in the art will recognize that the utilities herein described are not limited to

the specific experimental modes and materials presented and will appreciate the wider potential utility of this disclosure.

EXAMPLE 14

Recombinant Expression of VIAF

With the provision of several homologus VIAF cDNA sequences (SEQ ID NOs: 1, 3, 5, 7, and 9), the expression and purification of any VIAF protein homolog by standard laboratory techniques is now enabled. The purified protein may be used for functional analyses, antibody production, diagnosis, and therapy in a subject. Furthermore, the DNA sequence of the VIAF cDNA can be manipulated in studies to understand the expression of the gene and the function of its product. Mutant forms of VIAF may be isolated based upon information contained herein, and may be studied in order to detect alteration in expression patterns in terms of relative quantities, tissue specificity and functional properties of the encoded mutant VIAF protein. Partial or full-length cDNA sequences, which encode for the subject protein, may be ligated into bacterial expression vectors. Methods for expressing large amounts of protein from a cloned gene introduced into *E. coli* may be utilized for the purification, localization and functional analysis of proteins. For example, fusion proteins consisting of amino terminal peptides encoded by a portion of the *E. coli* lacZ or trpE gene linked to VIAF protein may be used to prepare polyclonal and monoclonal antibodies against this protein. Thereafter, these antibodies may be used to purify proteins by immunoaffinity chromatography, in diagnostic assays to quantitate the levels of protein and to localize proteins in tissues and individual cells by immunofluorescence.

Intact native protein may also be produced in *E. coli* in large amounts for functional studies. Methods and plasmid vectors for producing fusion proteins and intact native proteins in bacteria are described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, chapter 17, herein incorporated by reference). Such fusion proteins may be made in large amounts, are easy to purify, and can be used to elicit antibody response. Native proteins can be produced in bacteria by placing a strong, regulated promoter and an efficient ribosome binding site upstream of the cloned gene. If low levels of protein are produced, additional steps may be taken to increase protein production; if high levels of protein are produced, purification is relatively easy. Suitable methods are presented in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989) and are well known in the art. Often, proteins expressed at high levels are found in insoluble inclusion bodies. Methods for extracting proteins from these aggregates are described by Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, Chapter 17).

Vector systems suitable for the expression of lacZ fusion genes include the pUR series of vectors (Ruther and Muller-Hill, 1983, *EMBO J.* 2:1791), pEX1-3 (Stanley and Luzio, 1984, *EMBO J.* 3:1429) and pMR100 (Gray et al., 1982, *Proc. Natl. Acad. Sci. USA* 79:6598). Vectors

suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg, 1981, *Nature* 292:128), pKK177-3 (Amann and Brosius, 1985, *Gene* 40:183) and pET-3 (Studiar and Moffatt, 1986, *J. Mol. Biol.* 189:113). VIAF fusion proteins may be isolated from protein gels, lyophilized, ground into a powder and used as an antigen. The DNA sequence can also be transferred to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke et al., 1987, *Science* 236:806-12). These vectors may then be introduced into a variety of hosts including somatic cells, and simple or complex organisms, such as bacteria, fungi (Timberlake and Marshall, 1989, *Science* 244:1313-7), invertebrates, plants (Gasser and Fraley, 1989, Science 244:1293), and mammals (Pursel et al., 1989, *Science* 244:1281-8), which cell or organisms are rendered transgenic by the introduction of the heterologous VIAF cDNA.

For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus SV40, promoter in the pSV2 vector (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6), and introduced into cells, such as monkey COS-1 cells (Gluzman, 1981, *Cell* 23:175-82), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg, 1982, *J. Mol. Appl. Genet.* 1:327-41) and mycophoenolic acid (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6).

DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR.

The cDNA sequence (or portions derived from it) or a mini gene (a cDNA with an intron and its own promoter) may be introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the transcription of the cDNA eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the SV40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV40 are readily available (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6; Gorman *et al.*, 1982, *Proc. Natl. Acad. Sci USA* 78:6777-81). The level of expression of the cDNA can be manipulated with this type of vector, either by using promoters that have different activities (for example, the baculovirus pAC373 can express cDNAs at high levels in *S. frugiperda* cells (Summers and Smith, 1985, Genetically Altered Viruses and the Environment, Fields *et al.* (Eds.) 22:319-328, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.) or by using vectors that contain promoters amenable to modulation, for example, the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee *et al.*, 1982,

Nature 294:228). The expression of the cDNA can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

In addition, some vectors contain selectable markers such as the *gpt* (Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072-6) or *neo* (Southern and Berg, 1982, *J. Mol. Appl. Genet.* 1:327-41) bacterial genes. These selectable markers permit selection of transfected cells that exhibit stable, long-term expression of the vectors (and therefore the cDNA). The vectors can be maintained in the cells as episomal, freely replicating entities by using regulatory elements of viruses such as papilloma (Sarver *et al.*, 1981, *Mol. Cell Biol.* 1:486) or Epstein-Barr (Sugden *et al.*, 1985, *Mol. Cell Biol.* 5:410). Alternatively, one can also produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the cDNA as well) to create cell lines that can produce high levels of the gene product (Alt *et al.*, 1978, *J. Biol. Chem.* 253:1357).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells, is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, 1973, *Virology* 52:466) or strontium phosphate (Brash *et al.*, 1987, *Mol. Cell Biol.* 7:2013), electroporation (Neumann *et al.*, 1982, *EMBO J.* 1:841), lipofection (Felgner et al., 1987, *Proc. Natl. Acad. Sci USA* 84:7413), DEAE dextran (McCuthan *et al.*, 1968, *J. Natl. Cancer Inst.* 41:351), microinjection (Mueller *et al.*, 1978, *Cell* 15:579), protoplast fusion (Schafner, 1980, *Proc. Natl. Acad. Sci. USA* 77:2163-7), or pellet guns (Klein *et al.*, 1987, *Nature* 327:70). Alternatively, the cDNA can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein *et al.*, 1985, *Gen. Engrg.* 7:235), adenoviruses (Ahmad *et al.*, 1986, *J. Virol.* 57:267), or Herpes virus (Spaete et al., 1982, *Cell* 30:295).

These eukaryotic expression systems can be used for studies of the VIAF gene and mutant forms of this gene, the VIAF protein and mutant forms of this protein. Such uses include, for example, the identification of regulatory elements located in the 5' region of the VIAF gene on genomic clones that can be isolated from genomic DNA libraries, such as human or mouse libraries, using the information contained herein. The eukaryotic expression systems may also be used to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring or artificially produced mutant proteins. Naturally occurring mutant proteins may exist in a variety of cancers or diseases, while artificially produced mutant proteins can be designed by site directed mutagenesis as described herein. These latter studies may probe the function of any desired amino acid residue in the protein by mutating the nucleotide coding for that amino acid.

Using the above techniques, the expression vectors containing the VIAF gene or cDNA sequence or fragments or variants or mutants thereof can be introduced into human cells,

mammalian cells from other species or non-mammalian cells as desired. The choice of cell is determined by the purpose of the treatment. For example, monkey COS cells (Gluzman, 1981, *Cell* 23:175-82) that produce high levels of the SV40 T antigen and permit the replication of vectors containing the SV40 origin of replication may be used. Similarly, Chinese hamster ovary (CHO), mouse NIH 3T3 fibroblasts or human fibroblasts or lymphoblasts may be used.

One method that can be used to express the VIAF polypeptide from the cloned VIAF cDNA sequence in mammalian cells is to use the cloning vector pXTI (Stratagene). This vector contains the Long Terminal Repeats (LTRs) and a portion of the GAG gene from Moloney Murine Leukemia Virus. The position of the viral LTRs allows highly efficient, stable transfection of the region within the LTRs. The vector also contains the Herpes Simplex Thymidine Kinase promoter (TK), active in embryonal cells and in a wide variety of tissues in mice, and a selectable neomycin gene conferring G418 resistance. Two unique restriction sites, BglII and XhoI, are directly downstream from the TK promoter. VIAF cDNA, including the entire open reading frame for the VIAF protein and the 3' untranslated region of the cDNA is cloned into one of the two unique restriction sites downstream from the promoter.

The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. Positive transfectants are selected after growing the transfected cells in 600 μg/ml G418 (Sigma, St. Louis, MO). The protein is released into the supernatant and may be purified by standard immunoaffinity chromatography techniques using antibodies raised against the VIAF protein (see Examples 5 and 15).

Expression of VIAF protein in eukaryotic cells can be used as a source of proteins to raise antibodies. The VIAF protein may be extracted following release of the protein into the supernatant as described above, or, the cDNA sequence may be incorporated into a eukaryotic expression vector and expressed as a chimeric protein with, for example, β-globin. Antibody to β-globin is thereafter used to purify the chimeric protein. Corresponding protease cleavage sites engineered between the β-globin gene and the cDNA are then used to separate the two polypeptide fragments from one another after translation. One useful expression vector for generating β-globin chimeric proteins is pSG5 (Stratagene). This vector encodes rabbit β-globin.

The recombinant vector then contains the selected DNA of the DNA sequnces disclosed herein for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that the VIAF polypeptide can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be specifically selected from the group consisting of the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and

simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

The host cell, which may be transfected with the vector disclosed herein, may be selected from the group consisting of: *E. coli*, *Pseudomonas*, *B. subtilis*, *B. stearothermophilus* or other bacilli; other bacteria; yeast; fungi; plant; insect; mouse or other animal; or human tissue cells.

It is appreciated that for mutant or variant VIAF DNA sequences, similar systems are employed to express and produce the mutant or variant product.

EXAMPLE 15

Production of Anti-VIAF Antibodies

Monoclonal or polyclonal antibodies may be produced to any of the VIAF proteins herein disclosed, or variants, fragments and mutant forms thereof. Optimally, antibodies raised against VIAF would specifically detect the VIAF protein. That is, such antibodies would recognize and bind the protein and would not substantially recognize or bind to other proteins found in human or other cells. The determination that an antibody specifically detects the VIAF protein is made by any one of a number of standard immunoassay methods; for instance, the Western blotting technique (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). To determine that a given antibody preparation (such as one produced in a mouse against VIAF) specifically detects the VIAF protein by Western blotting, total cellular protein is extracted from cells (for example, lymphocytes) and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. The proteins are then transferred to a membrane (for example, nitrocellulose) by Western blotting, and the antibody preparation is incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies is detected by the use of an anti-mouse antibody conjugated to an enzyme such as alkaline phosphatase; application of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium results in the production of a dense blue compound by immuno-localized alkaline phosphatase.

Antibodies which specifically detect the VIAF protein will, by this technique, be shown to bind to the VIAF protein band (which will be localized at a given position on the gel determined by its molecular weight). Non-specific binding of the antibody to other proteins may occur and may be detectable as a weak signal on the Western blot. The non-specific nature of this binding will be recognized by one skilled in the art by the weak signal obtained on the Western blot relative to the strong primary signal arising from the specific antibody-VIAF protein binding.

Substantially pure VIAF protein suitable for use as an immunogen is isolated as already described. Concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms per milliliter. Monoclonal or polyclonal antibody to the protein can then be prepared.

Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes of the VIAF protein can be identified, isolated and prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495, 1975) or derivative methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein over a period of a few weeks. The mouse is then sacrificed, and the antibody-producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall (*Enzymol.* 70:419, 1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Harlow and Lane (Antibodies: A Laboratory Manual. 1988, Cold Spring Harbor Laboratory, New York). In addition, protocols for producing humanized forms of monoclonal antibodies (for therapeutic applications) and fragments of monoclonal antibodies are known in the art.

Polyclonal Antibody Production by Immunization

Polyclonal antiserum containing antibodies to heterogeneous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein (for example see EXAMPLES 6 and 14), which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis et al. (*J. Clin. Endocrinol. Metab.* 33:988-91, 1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony et al. (In: *Handbook of Experimental Immunology*, Wier, D. (ed.). Chapter 19. Blackwell. 1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher (*Manual of Clinical Immunology*, Chapter 42. 1980).

Antibodies Raised against Synthetic Peptides

Another approach to raising antibodies against VIAF is to use synthetic peptides synthesized on a commercially available peptide synthesizer based upon the predicted amino acid sequence of the VIAF protein, for example SEQ ID NOs 2, 4, 6, 8 and 10. The chemical synthesis described in EXAMPLE 23 for example may be used to generate a synthetic VIAF protein.

Antibodies Raised by Injection of VIAF cDNA

Antibodies may be raised against the VIAF protein by subcutaneous injection of a DNA vector which expresses the VIAF protein into laboratory animals, such as mice. Delivery of the recombinant vector into the animals may be achieved using a hand-held form of the Biolistic system (Sanford *et al.*, *Particulate Sci. Technol.* 5:27-37, 1987) as described by Tang *et al.* (*Nature* 356:152-4, 1992). Expression vectors suitable for this purpose may include those which express the VIAF cDNA under the transcriptional control of either the human β-actin promoter or the cytomegalovirus (CMV) promoter.

Antibody preparations prepared according to these protocols are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample.

Labeled Antibodies

Antibodies disclosed herein can be conjugated with various labels for their direct detection (see Chapter 9, Harlow and Lane, *Antibodies: A Laboratory Manual*. 1988). The label, which may include, but is not limited to, a radiolabel, enzyme, fluorescent probe, or biotin, is chosen based on the method of detection available to the user.

Antibodies can be radiolabeled with iodine (^{125}I), which yields low-energy gamma and X-ray radiation. Briefly, 10 μg of protein in 25 μl of 0.5 M sodium phosphate (pH 7.50 is placed in a 1.5 ml conical tube. To this, 500 μC of $Na^{125}I$, and 25 μl of 2 mg/ml chloramine T is added and incubated for 60 seconds at room temperature. To stop the reaction, 50μl of chloramine T stop buffer is added (2.4 mg/ml sodium metabisulfite, 10 mg/ml tyrosine, 10% glycerol, 0.1% xylene cyanol in PBS). The iodinated antibody is separated from the iodotyrosine on a gel filtration column. Antibodies disclosed herein can also be labeled with biotin, with enzymes such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) or with fluorescent dyes. The method of producing these conjugates is determined by the reactive group on the label added.

EXAMPLE 16

Diagnostic Methods

An embodiment disclosed herein is a method for screening a subject to determine if the subject carries a mutant or variant VIAF gene, for example having a heterozygous or homozygous nucleotide change, or insertions or deletions of the VIAF gene, including partial or complete deletion of the gene. One major application of the VIAF sequence information presented herein is in the area of genetic testing for predisposition to disease, such as: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; 6-12 leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes, owing to a VIAF deletion or mutation. The gene sequence of the VIAF genes, including intron-exon boundaries is also useful in such diagnostic methods. The method consists of providing a biological sample obtained from the subject, in which the sample includes DNA or RNA, and providing an assay for detecting in the biological sample the presence of a mutant VIAF gene, a mutant VIAF RNA, a homozygously or heterozygously deleted VIAF gene, or the absence, through deletion, of the VIAF gene and corresponding RNA. Suitable biological samples include samples obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, fine needle aspirate specimen, amniocentesis samples and autopsy material. The detection in the biological sample may be performed by a number of methodologies, as outlined below.

The foregoing assay may be assembled in the form of a diagnostic kit and may include, for example: hybridization with oligonucleotides; PCR amplification of the gene or a part thereof using oligonucleotide primers; RT-PCR amplification of the RNA or a part thereof using oligonucleotide primers; or direct sequencing of the VIAF gene of the subject's genome using oligonucleotide primers. The efficiency of these molecular genetic methods should permit a rapid classification of patients affected by mutations, deletions or variants of the VIAF gene.

One embodiment of such detection techniques is the polymerase chain reaction amplification of reverse transcribed RNA (RT-PCR) of RNA isolated from cells (for example lymphocytes) followed by direct DNA sequence determination of the products. The presence of one or more nucleotide differences between the obtained sequence and the cDNA sequences, and especially, differences in the ORF portion of the nucleotide sequence are taken as indicative of a potential VIAF gene mutation.

Alternatively, DNA extracted from lymphocytes or other cells may be used directly for amplification. The direct amplification from genomic DNA would be appropriate for analysis of the entire VIAF gene including regulatory sequences located upstream and downstream from the open reading frame. Recent reviews of direct DNA diagnosis have been presented by Caskey (*Science* 236:1223-1228, 1989) and by Landegren et al. (*Science* 242:229-37, 1989).

Further studies of VIAF genes isolated from subjects may reveal particular mutations, deletions, or variants which occur at a high frequency within this population of individuals. In this case, rather than sequencing the entire VIAF gene, it is possible to design DNA diagnostic methods to specifically detect the most common VIAF mutations, deletions, or variants.

The detection of specific DNA mutations may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 51:257-61), direct DNA sequencing (Church and Gilbert, 1984, *Proc. Natl. Acad. Sci. USA*. 81:1991-5), the use of restriction enzymes (Flavell et al., 1978, *Cell* 15:25; Geever et al., 1981, *Proc. Natl. Acad. Sci USA* 78:5081), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis, 1986, *Cold Spring Harbor Symp. Quant. Biol.* 51:275-284), RNase protection (Myers et al., 1985, *Science* 230:1242), chemical cleavage (Cotton et al., 1985, *Proc. Natl. Acad. Sci. USA* 85:4397-401), and the ligase-mediated detection procedure (Landegren et al., 1988, *Science* 241:1077).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labeled radioactively with isotopes (such as ^{32}P) or non-radioactively, with tags such as biotin (Ward and Langer et al., 1981. *Proc. Natl. Acad. Sci. USA* 78:6633-57), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence of these specific sequences are visualized by methods such as autoradiography or fluorometric (Landegren et al., 1989, *Science* 242:229-37) or colorimetric reactions (Gebeyehu et al., 1987, *Nucleic Acids Res.* 15:4513-34). The absence of hybridization would indicate a mutation in the particular region of the gene, or a deleted VIAF gene.

Sequence variations between normal and mutant forms of the VIAF gene may also be revealed by the direct DNA sequencing method of Church and Gilbert (*Proc. Natl. Acad. Sci. USA* 81:1991-5, 1988). Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR (Wrichnik et al., 1987, *Nucleic Acids Res.* 15:529-42; Wong et al., 1987, *Nature* 330:384-6; Stoflet et al., 1988, *Science* 239:491-4). In this approach, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence alterations may occasionally generate fortuitous restriction enzyme recognition sites or may eliminate existing restriction sites. Changes in restriction sites are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization (Southern, 1975, *J. Mol. Biol.* 98:503). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme;

fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high-resolution gel electrophoresis. For example, a PCR product with small deletions is clearly distinguishable from a normal sequence on an 8% non-denaturing polyacrylamide gel (WO 91/10734; Nagamine et al., 1989, *Am. J. Hum. Genet.* 45:337-9). DNA fragments of different sequence compositions may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperatures (Myers et al., 1985, *Science* 230:1242). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length in a PCR. For example, an invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.

In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments can be visualized by methods where individual DNA samples are not immobilized on membranes. The probe and target sequences may be both in solution, or the probe sequence may be immobilized (Saiki *et al.*, 1989, *Proc. Nat. Acad. Sci. USA* 86:6230-4). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving calorigenic reactions and fluorometry involved fluorogenic reactions, may be used to identify specific individual genotypes.

If more than one mutation is frequently encountered in the VIAF gene, a system capable of detecting such multiple mutations is desirable. For example, a PCR with multiple, specific oligonucleotide primers and hybridization probes may be used to identify all possible mutations at the same time (Chamberlain *et al.*, 1988, *Nucl. Acids Res.* 16:1141-55). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki *et al.*, 1989, *Proc. Nat. Acad. Sci. USA* 86:6230-4).

EXAMPLE 17

Quantitation of VIAF Proteins

An alternative method of diagnosing a VIAF gene deletion, variant, or other mutation is to quantitate the level of VIAF protein in the cells of a subject. This diagnostic tool is useful for detecting reduced levels of the VIAF protein which result from, for example, mutations in the promoter regions of the VIAF gene or mutations within the coding region of the gene which produced truncated, non-functional polypeptides, as well as from deletions of the entire VIAF gene.

These diagnostic methods, in addition to those described in EXAMPLE 16, provide an enhanced ability to diagnose susceptibility to diseases caused by mutation or deletion of these genes.

The determination of reduced VIAF protein levels would be an alternative or supplemental approach to the direct determination of VIAF gene deletion or mutation status by the methods outlined above in EXAMPLE 16. The availability of antibodies specific to the VIAF protein (for example those described in Examples 5 and 15) will facilitate the quantitation of cellular VIAF protein by one of a number of immunoassay methods which are well known in the art and are presented in Harlow and Lane (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York. 1988).

Such assays permit both the detection of VIAF proteins in a biological sample and the quantitation of such proteins. Typical methods involve: providing a biological sample of the subject in which the sample contains cellular proteins, and providing an immunoassay for quantitating the level of VIAF protein in the biological sample. This can be achieved by combining the biological sample with a VIAF specific binding agent, such as an anti-VIAF antibody (such as monoclonal or polyclonal antibodies), so that complexes form between the binding agent and the VIAF protein present in the sample, and then detecting or quantitating such complexes.

In particular forms, these assays may be performed with the VIAF specific binding agent immobilized on a support surface, such as in the wells of a microtiter plate or on a column. The biological sample is then introduced onto the support surface and allowed to interact with the specific binding agent so as to form complexes. Excess biological sample is then removed by washing, and the complexes are detected with a reagent, such as a second anti-VIAF protein antibody that is conjugated with a detectable marker.

In an alternative assay, the cellular proteins are isolated and subjected to SDS-PAGE followed by Western blotting, for example as described in EXAMPLE 6. After resolving the proteins, the proteins are transferred to a membrane, which is probed with specific binding agents that recognize VIAF. The proteins are detected, for example with HRP-conjugated secondary antibodies, and quantitated.

In yet another assay, the level of VIAF protein in cells is analyzed using microscopy. Using specific binding agents which recognize VIAF, samples can be analyzed for the presence of VIAF proteins. For example, frozen biopsied tissue sections are thawed at room temperature and fixed with acetone at -200°C for five minutes. Slides are washed twice in cold PBS for five minutes each, then air-dried. Sections are covered with 20-30 μl of antibody solution (15-45 μg/ml) (diluted in PBS, 2% BSA at 15-50 μg/ml) and incubated at RT in a humidified chamber for 30 minutes. Slides are washed three times with cold PBS five minutes each, allowed to air-dry briefly (5 minutes) before applying 20-30 μl of the second antibody solution (diluted in PBS, 2% BSA at 15-50 μg/ml) and incubated at room temperature in humidified chamber for 30 minutes. The label on the second antibody may contain a fluorescent probe, enzyme, radiolabel, biotin, or

other detectable marker. The slides are washed three times with cold PBS 5 minutes each then quickly dipped in distilled water, air-dried, and mounted with PBS containing 30% glycerol. Slides can be stored at 4°C prior to viewing.

For samples prepared for electron microscopy (versus light microscopy), the second antibody is conjugated to gold particles. Tissue is fixed and embedded with epoxy plastics, then cut into very thin sections (~1-2 μm). The specimen is then applied to a metal grid, which is then incubated in the primary anti-VIAF antibody, washed in a buffer containing BSA, then incubated in a secondary antibody conjugated to gold particles (usually 5-20 nm). These gold particles are visualized using electron microscopy methods.

For the purposes of quantitating the VIAF proteins, a biological sample of the subject, which sample includes cellular proteins, is required. Such a biological sample may be obtained from body cells, such as those present in which expression of the protein has been detected. As shown in FIG. 3, for example, VIAF could be analyzed in cells isolated from the testis, ovary or prostate, but its expression in peripheral blood leukocytes is clearly the most accessible and convenient source from which specimens can be obtained. Specimens can be obtained from peripheral blood, urine, saliva, tissue biopsy, amniocentesis samples, surgical specimens, fine needle aspirates, and autopsy material, particularly cancer cells. Quantitation of VIAF proteins would be made by immunoassay and compared to levels of the protein found in non-VIAF expressing human cells or to the level of VIAF in healthy cells (cells of the same origin that are not neoplastic). A significant (for example 50% or greater) reduction in the amount of VIAF protein in the cells of a subject compared to the amount of VIAF protein found in non-VIAF expressing cells or that found in normal cells, would be taken as an indication that the subject may have deletions or mutations in the VIAF gene locus.

EXAMPLE 18

Gene Therapy

A new gene therapy approach for subjects suffering from VIAF gene deletions or mutations is now made possible by the present disclosure. Essentially, cells may be removed from a subject having deletions or mutations of the VIAF gene, and then transfected with an expression vector containing VIAF cDNA. These transfected cells will thereby produce functional VIAF protein and can be reintroduced into the subject.

The scientific and medical procedures required for human cell transfection are now routine procedures. The provision herein of VIAF cDNAs now allows the development of human gene therapy based upon these procedures. Immunotherapy of melanoma patients using genetically engineered tumor-infiltrating lymphocytes (TILs) has been reported by Rosenberg *et al.* (*N. Engl. J. Med.* 323:570-8, 1990). In that study, a retrovirus vector was used to introduce a gene for

neomycin resistance into TILs. A similar approach may be used to introduce the VIAF cDNA into patients affected by VIAF deletions or mutations.

In some embodiments, a method of treating tumors which underexpress VIAF, or in which greater VIAF expression is desired, is disclosed. These methods can be accomplished by introducing a gene coding for VIAF into a subject. A general strategy for transferring genes into donor cells is disclosed in U.S. Patent No. 5,529,774, incorporated by reference. Generally, a gene encoding a protein having therapeutically desired effects is cloned into a viral expression vector, and that vector is then introduced into the target organism. The virus infects the cells, and produces the protein sequence *in vivo*, where it has its desired therapeutic effect. See, for example, Zabner *et al.* (*Cell* 75:207-16, 1993).

In some of the foregoing examples, it may only be necessary to introduce the genetic or protein elements into certain cells or tissues. For example, in the case of benign nevi and psoriasis, introducing them into only the skin may be sufficient. However, in some instances (i.e. tumors and polycythemia inflammatory fibrosis), it may be more therapeutically effective and simple to treat all of the patients cells, or more broadly disseminate the vector, for example by intravascular administration.

The nucleic acid sequence encoding at least one therapeutic agent is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, the gene's native promoter, retroviral LTR promoter, or adenoviral promoters, such as the adenoviral major late promoter; the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMTV promoter; the metallothionein promoter; heat shock promoters; the albumin promoter; the histone promoter; the α-actin promoter; TK promoters; B19 parvovirus promoters; and the ApoAI promoter. However the scope of the disclosure is not limited to specific foreign genes or promoters.

The recombinant nucleic acid can be administered to the subject by any method which allows the recombinant nucleic acid to reach the appropriate cells. These methods include injection, infusion, deposition, implantation, or topical administration. Injections can be intradermal or subcutaneous. The recombinant nucleic acid can be delivered as part of a viral vector, such as avipox viruses, recombinant vaccinia virus, replication-deficient adenovirus strains or poliovirus, or as a non-infectious form such as naked DNA or liposome encapsulated DNA.

EXAMPLE 19

Viral Vectors for Gene Therapy

Adenoviral vectors may include essentially the complete adenoviral genome (Shenk *et al.*, *Curr. Top. Microbiol. Immunol.* 111:1-39, 1984). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted. In one embodiment, the vector includes an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral

encapsidation signal; a DNA sequence encoding a therapeutic agent; and a promoter for expressing the DNA sequence encoding a therapeutic agent. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not necessarily free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins transcribed by the adenoviral major late promoter. In another embodiment, the vector may be an adeno-associated virus (AAV) such as described in U.S. Patent No. 4,797,368 (Carter *et al.*) and in McLaughlin et al. (*J. Virol.* 62:1963-73, 1988) and AAV type 4 (Chiorini *et al. J. Virol.* 71:6823-33, 1997) and AAV type 5 (Chiorini *et al. J. Virol.* 73:1309-19, 1999)

Such a vector may be constructed according to standard techniques, using a shuttle plasmid which contains, beginning at the 5' end, an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a tripartite leader sequence, a multiple cloning site (which may be as herein described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The DNA segment serves as a substrate for homologous recombination with a modified or mutated adenovirus, and may encompass, for example, a segment of the adenovirus 5' genome no longer than from base 3329 to base 6246. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. A desired DNA sequence encoding a therapeutic agent may be inserted into the multiple cloning site of the plasmid.

The plasmid may be used to produce an adenoviral vector by homologous recombination with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell line, such as 293 cells, by $CaPO_4$ precipitation. The homologous recombination produces a recombinant adenoviral vector which includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the adenovirus may be constructed by using a yeast artificial chromosome (or YAC) containing an adenoviral genome according to the method described in Ketner et al. (*Proc. Natl. Acad. Sci. USA*, 91:6186-90, 1994), in conjunction with the teachings contained herein. In this embodiment, the adenovirus yeast artificial chromosome is produced by homologous recombination *in vivo* between adenoviral DNA and yeast artificial chromosome plasmid vectors carrying segments of the adenoviral left and right genomic termini. A DNA sequence encoding a therapeutic agent then may be cloned into the adenoviral DNA. The modified adenoviral genome then is excised from the adenovirus yeast artificial chromosome in order to be used to generate adenoviral vector particles as hereinabove described.

The adenoviral particles are administered in an amount effective to produce a therapeutic effect in a subject. The exact dosage of adenoviral particles to be administered is dependent upon a variety of factors, including the age, weight, and sex of the subject to be treated, and the nature and extent of the disease or disorder to be treated. The adenoviral particles may be administered as part of a preparation having a titer of adenoviral particles of at least 1 x 10^{10} pfu/ml, and in general not exceeding 2 x 10^{11} pfu/ml. The adenoviral particles may be administered in combination with a pharmaceutically acceptable carrier in a volume up to 10 ml. The pharmaceutically acceptable carrier may be, for example, a liquid carrier such as a saline solution, protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ), or Polybrene (Sigma Chemical) as well as those described in EXAMPLE 24.

In another embodiment, the viral vector is a retroviral vector. Retroviruses have been considered for experiments in gene therapy because they have a high efficiency of infection and stable integration and expression (Orkin *et al.*, 1988, *Prog. Med. Genet.*7:130-42). The full length VIAF gene or cDNA can be cloned into a retroviral vector and driven from either its endogenous promoter or from the retroviral LTR (long terminal repeat). Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. The vector is generally a replication defective retrovirus particle.

Retroviral vectors are useful as agents to effect retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

Other viral transfection systems may also be utilized for this type of approach, including Vaccinia virus (Moss *et al.*, 1987, *Annu. Rev. Immunol.* 5:305-24), Bovine Papilloma virus (Rasmussen *et al.*, 1987, *Methods Enzymol.* 139:642-54) or members of the herpes virus group such as Epstein-Barr virus (Margolskee *et al.*, 1988, *Mol. Cell. Biol.* 8:2837-47). Recent developments in gene therapy techniques include the use of RNA-DNA hybrid oligonucleotides, as described by Cole-Strauss *et al.* (*Science* 273:1386-9, 1996). This technique can allow for site-specific integration of cloned sequences, permitting accurately targeted gene replacement.

New genes may be incorporated into proviral backbones in several general ways. In the most straightforward constructions, the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more

than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter. Alternatively, two genes may be expressed from a single promoter by the use of an Internal Ribosome Entry Site.

EXAMPLE 20

Two Step Assay to Detect the Presence of VIAF Gene in a Sample

A tissue sample from a subject is processed according to the method disclosed by Antonarakis *et al.* (*New Eng. J. Med.* 313:842-848, 1985), separated through a 1% agarose gel and transferred to a nylon membrane for Southern blot analysis. Membranes are UV cross linked at 150 mJ using a GS Gene Linker (Bio-Rad). A VIAF probe (for example, SEQ ID No: 1) is subcloned into pTZ18U. The phagemids are transformed into *E. coli* MV 1190 infected with M13KO7 helper phage (Bio-Rad, Richmond, Calif.). Single stranded DNA is isolated according to standard procedures (Sambrook, *et al. Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989).

Blots are prehybridized for 15-30 minutes at 65°C in 7% sodium dodecyl sulfate (SDS) in 0.5 M $NaPO_4$. The methods follow those described by Nguyen *et al.* (*BioTechniques* 13:116-23, 1992). The blots are hybridized overnight at 65°C in 7% SDS, 0.5 M $NaPO_4$ with 25-50 ng/ml single stranded probe DNA. Post-hybridization washes consist of two 30 minute washes in 5% SDS, 40 mM $NaPO_4$ at 65°C, followed by two 30-minute washes in 1% SDS, 40 mM $NaPO_4$ at 65°C.

The blots are subsequently rinsed with phosphate buffered saline (pH 6.8) for five minutes at RT and incubated with 0.2% casein in PBS for five minutes. The blots are then preincubated for 5-10 minutes in a shaking water bath at 45°C with hybridization buffer consisting of 6 M urea, 0.3 M NaCl, and 5X Denhardt's solution (see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). The buffer is removed and replaced with 50-75 $\mu l/cm^2$ fresh hybridization buffer plus 2.5 nM of the covalently cross-linked oligonucleotide sequence complementary to the universal primer site (UP-AP, Bio-Rad). The blots are hybridized for 20-30 minutes at 45°C and post hybridization washes are incubated at 45°C as two 10 minute washes in 6 M urea, 1X standard saline citrate (SSC), 0.1% SDS and one 10 minute wash in 1XSSC, 0.1% Triton™X-100. The blots are rinsed for 10 minutes at RT with 1XSSC.

Blots are incubated for 10 minutes at RT with shaking in the substrate buffer consisting of 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide, pH 10.0. Individual blots are placed in heat sealable bags with substrate buffer and 0.2 mM AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt, Bio-Rad). After a 20 minute incubation at RT with shaking, the excess AMPPD solution is removed. The blot is exposed to X-ray film overnight. Positive bands indicate the presence of the VIAF gene. Patient samples which show no

hybridizing bands lack the VIAF gene, indicating the possibility of ongoing disease such as cancer, or an enhanced susceptibility to developing a disease, such as cancer, in the future.

EXAMPLE 21

Peptide Modifications

Also disclosed are biologically active molecules that mimic the action (mimetics) of the VIAF proteins, fragments, variants and mutants disclosed herein. Synthetic embodiments of naturally-occurring peptides described herein, as well as analogues (non-peptide organic molecules), derivatives (chemically functionalized peptide molecules obtained starting with the disclosed peptide sequences) and variants (homologs) of these peptides that specifically induce apoptosis. Each peptide ligand disclosed herein is comprised of a sequence of amino acids, which may be either L- and/or D- amino acids, naturally occurring and otherwise.

Peptides may be modified by a variety of chemical techniques to produce derivatives having essentially the same activity as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the peptide, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C1-C16 ester, or converted to an amide of formula NR1R2 wherein R1 and R2 are each independently H or C1-C16 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6- membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C1-C16 alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the peptide side chain may be converted to C1-C16 alkoxy or to a C1-C16 ester using well-recognized techniques. Phenyl and phenolic rings of the peptide side chain may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with C1-C16 alkyl, C1-C16 alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide sidechains can be extended to homologous C2-C4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides disclosed herein to select and provide conformational constraints to the structure that result in enhanced stability. For example, a carboxyl-terminal or amino-terminal cysteine residue can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, thereby generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

To maintain an optimally functional peptide, particular peptide variants will differ by only a small number of amino acids from the peptides disclosed in this specification. Such variants may have deletions (for example of 1-3 or more amino acid residues), insertions (for example of 1-3 or

more residues), or substitutions that do not interfere with the desired activity of the peptides. Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. For example, such variants can have amino acid substitutions of single residues, for example 1, 3, 5 or even 10 substitutions in the full length VIAF protein (SEQ ID NOS 2, 4, 6, 8 and 10).

Peptidomimetic and organomimetic embodiments are also disclosed herein, whereby the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid side chains in the peptide, resulting in such peptido- and organomimetics of the peptides having substantial specific hair growth promoting and blocking activity. For computer modeling applications, a pharmacophore is an idealized, three-dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modeling software (using computer assisted drug design or CADD). See Walters, "Computer-Assisted Modeling of Drugs", in Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press: Buffalo Grove, IL, pp. 165-174 and Principles of Pharmacology (ed. Munson, 1995), chapter 102 for a description of techniques used in CADD. Also disclosed are mimetics prepared using such techniques that produce either peptides or conventional organic pharmaceuticals that retain the biological activity of VIAF.

The above described mimetics are examined for their ability to modulate the anti-apoptotic and signaling properties of the IAP family. Such activities can be readily determined using the assays disclosed herein, for example using the methods described in EXAMPLES 7-10. Suitable mimetics would demonstrate VIAF biological activity as defined above.

EXAMPLE 22

Method for Generating Mimetics

Compounds or other molecules which mimic normal VIAF function, such as compounds which modulate the anti-apoptotic and signaling properties of the IAP family, can be identified and/or designed. These compounds or molecules are known as mimetics, because they mimic the biological activity of the normal protein.

Crystallography

To identify the amino acids that interact between the IAPs and VIAF, VIAF is co-crystallized in the presence of an IAP protein, for example, XIAP, c-IAP1 or c-IAP2. One method that can be used is the hanging drop method. In this method, a concentrated salt, IAP and VIAF protein solution is applied to the underside of a lid of a multiwell dish. A range of concentrations may need to be tested. The lid is placed onto the dish, such that the droplet "hangs" from the lid. As the solvent evaporates, a protein crystal is formed, which can be visualized with a microscope.

This crystallized structure is then subjected to X-ray diffraction or NMR analysis which allows for the identification of the amino acid residues that are in contact with one another. The amino acids that contact the transcription factors establish a pharmacophore that can then be used to identify drugs that interact at that same site.

Identification of drugs

Once these amino acids have been identified, one can screen synthetic drug databases (which can be licensed from several different drug companies), to identify drugs that interact with the same amino acids of VIAF that IAPs, such as XIAP, c-IAP1 or c-IAP2, interact with. Moreover, structure activity relationships and computer assisted drug design can be performed as described in Remington, *The Science and Practice of Pharmacy*, Chapter 28.

Designing synthetic peptides

In addition, synthetic peptides can be designed from the sequence of an IAP (such as XIAP, cIAP-1 or cIAP-2) that interacts with VIAF. Several different peptides could be generated from this region. This could be done with or without the crystallography data. However, once crystallography data is available, peptides can also be designed that bind better than VIAF.

The chimeric peptides may be expressed recombinantly, for example in *E. coli*. One advantage of the synthetic peptides over the monoclonal antibodies is that they are smaller, and therefore diffuse easier, and are not as likely to be immunogenic. Standard mutagenesis of such peptides can also be performed to identify variant peptides having even greater enhancement of transcription and splicing.

After synthetic drugs or peptides that bind to IAPs have been identified, their ability to modulate the anti-apoptotic and signaling properties of the IAP family, can be tested as described in the above EXAMPLES 7-10. Those that are positive would be good candidates for therapies, such as treatment of diseases including, but not limited to: cancer; autoimmune diseases such as diabetes and multiple sclerosis; neurodegenerative diseases including retinal degeneration; heterocellular hereditary persistence of fetal hemoglobin; deafness; cardiomyopathy; 6-12 leukemia; diabetes mellitus, transient neonatal; and insulin dependent diabetes, or diseases in which VIAF is underexpressed VIAF or where greater expression of VIAF is desired.

EXAMPLE 23

Peptide Synthesis and Purification

The disclosed peptides (and variants, and fragments, and mutants thereof) can be chemically synthesized by any of a number of manual or automated methods of synthesis known in the art. For example, solid phase peptide synthesis (SPPS) is carried out on a 0.25 millimole (mmole) scale using an Applied Biosystems Model 431A Peptide Synthesizer and using 9-

fluorenylmethyloxycarbonyl (Fmoc) amino-terminus protection, coupling with dicyclohexylcarbodiimide/ hydroxybenzotriazole or 2-(1H-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/ hydroxybenzotriazole (HBTU/HOBT), and using p-hydroxymethylphenoxymethylpolystyrene (HMP) or Sasrin resin for carboxyl-terminus acids or Rink amide resin for carboxyl-terminus amides.

Fmoc-derivatized amino acids are prepared from the appropriate precursor amino acids by tritylation and triphenylmethanol in trifluoroacetic acid, followed by Fmoc derivitization as described by Atherton *et al.* (*Solid Phase Peptide Synthesis*, IRL Press: Oxford, 1989).

Sasrin resin-bound peptides are cleaved using a solution of 1% TFA in dichloromethane to yield the protected peptide. Where appropriate, protected peptide precursors are cyclized between the amino- and carboxyl-termini by reaction of the amino-terminal free amine and carboxyl-terminal free acid using diphenylphosphorylazide in nascent peptides wherein the amino acid sidechains are protected.

HMP or Rink amide resin-bound products are routinely cleaved and protected sidechain-containing cyclized peptides deprotected using a solution comprised of trifluoroacetic acid (TFA), optionally also comprising water, thioanisole, and ethanedithiol, in ratios of 100 : 5 : 5 : 2.5, for 0.5 - 3 hours at RT.

Crude peptides are purified by preparative high pressure liquid chromatography (HPLC), for example using a Waters Delta-Pak C18 column and gradient elution with 0.1% TFA in water modified with acetonitrile. After column elution, acetonitrile is evaporated from the eluted fractions, which are then lyophilized. The identity of each product so produced and purified may be confirmed by fast atom bombardment mass spectroscopy (FABMS) or electrospray mass spectroscopy (ESMS).

EXAMPLE 24

Pharmaceutical Compositions and Modes of Administration

Various delivery systems for administering the combined therapy disclosed herein are known, and include e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see Wu and Wu, *J. Biol. Chem.* 1987, 262:4429-32), and construction of a therapeutic nucleic acid as part of a retroviral or other vector. Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, the pharmaceutical compositions may be introduced into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular

injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

In one embodiment, it may be desirable to administer the pharmaceutical compositions disclosed herein locally to the area in need of treatment, for example, by local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, through a catheter, by a suppository or an implant, such as a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

The use of liposomes as a delivery vehicle is one delivery method of interest. The liposomes fuse with the target site and deliver the contents of the lumen intracellularly. The liposomes are maintained in contact with the target cells for a sufficient time for fusion to occur, using various means to maintain contact, such as isolation and binding agents. Liposomes may be prepared with purified proteins or peptides that mediate fusion of membranes, such as Sendai virus or influenza virus. The lipids may be any useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine. Other potential lipids include neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like. For preparing the liposomes, the procedure described by Kato *et al.* (*J. Biol. Chem.* 1991, 266:3361) may be used.

The present disclosure also provides pharmaceutical compositions which include a therapeutically effective amount of the VIAF protein, RNA, DNA, antisense molecule or antibody, alone or with a pharmaceutically acceptable carrier.

Delivery systems

Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile, and the formulation suits the mode of administration. The composition can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate.

The amount of the inducing agent and disrupting agent that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease

or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The disclosure also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. Instructions for use of the composition can also be included.

The pharmaceutical compositions or methods of treatment may be administered in combination with other therapeutic treatments, such as other antineoplastic or antitumorigenic therapies.

Administration of Nucleic Acid Molecules

In an embodiment in which a VIAF nucleic acid is employed for gene therapy, the analog is delivered intracellularly (e.g., by expression from a nucleic acid vector or by receptor-mediated mechanisms). In an embodiment where the therapeutic molecule is a nucleic acid or antisense molecule, administration can be achieved by an appropriate nucleic acid expression vector which is administered so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot *et al.*, *Proc. Natl. Acad. Sci. USA* 1991, 88:1864-8), etc. Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The vector pcDNA, is an example of a method of introducing the foreign cDNA into a cell under the control of a strong viral promoter (CMV) to drive the expression. However, other vectors can be used (see EXAMPLES 14, 18, and 19). Other retroviral vectors (such as pRETRO-ON, Clontech), also use this promoter but have the advantages of entering cells without any transfection aid, integrating into the genome of target cells ONLY when the target cell is dividing (as cancer cells do, especially during first remissions after chemotherapy) and they are regulated. It is also possible to turn on the expression of the VIAF nucleic acid by administering tetracycline when these plasmids are used. Hence these plasmids can be allowed to transfect the cells, then administer a course of tetracycline with a course of chemotherapy to achieve better cytotoxicity.

Other plasmid vectors, such as pMAM-neo (Clontech) or pMSG (Pharmacia) use the MMTV-LTR promoter (which can be regulated with steroids) or the SV10 late promoter (pSVL, Pharmacia) or metallothionein-responsive promoter (pBPV, Pharmacia) and other viral vectors,

including retroviruses. Examples of other viral vectors include adenovirus, AAV (adeno-associated virus), recombinant HSV, poxviruses (vaccinia) and recombinant lentivirus (such as HIV). These vectors achieve the basic goal of delivering into the target cell the cDNA sequence and control elements needed for transcription. The present disclosure includes all forms of nucleic acid delivery, including synthetic oligos, naked DNA, plasmid and viral, integrated into the genome or not.

Administration of Antibodies

In an embodiment where the therapeutic molecule is an antibody, specifically an antibody that recognizes VIAF protein, administration may be achieved by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents. Similar methods can be used to administer VIAF protein, of fragments thereof.

The disclosure also provides pharmaceutical compositions which include a therapeutically effective amount of the antibody, and a pharmaceutically acceptable carrier or excipient.

EXAMPLE 25

Disruption of VIAF Expression

This example describes methods that can be used to disrupt VIAF expression. Such methods are useful when apoptosis is desired, for example in the pathogenesis of proliferative disorders, for example neoplasms, such as cancer. In addition, in *in vitro* systems, it has been shown that apoptosis is used to fight viral infections. Furthermore, apoptosis is necessary for normal embryonal development. One approach to disrupting VIAF function or expression is to use antisense oligonucleotides.

To design an antisense oligonucleotide, the mRNA sequence from the desired molecule, such as human VIAF, is examined. Regions of the sequence containing multiple repeats, such as TTTTTTTT, are not as desirable because they will lack specificity. Several different regions can be chosen. Of those, oligos are selected by the following characteristics: ones having the best conformation in solution; ones optimized for hybridization characteristics; and one having less potential to form secondary structures. Antisense molecules having a propensity to generate secondary structures are less desirable.

Plasmids containing VIAF antisense sequences can also be genereated. For example, cDNA fragments coding for human VIAF are PCR amplified. The nucleotides are then amplified using Pfu DNA polymerase (Stratagene) and cloned in antisense orientation a vector, such as pcDNA vectors (InVitrogen, Carlsbad CA). The nucleotide sequence and orientation of the insert can be confirmed by dideoxy sequencing using a Sequenase kit (Amersham Pharmacia Biotech).

Generally, the term "antisense" refers to a nucleic acid capable of hybridizing to a portion of a VIAF RNA (such as mRNA) by virtue of some sequence complementarity. The antisense nucleic acids disclosed herein can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

The VIAF antisense nucleic acids are polynucleotides, and may be oligonucleotides (ranging from 6 to about 100 oligonucleotides). In specific aspects, the oligonucleotide is at least 10, 15, or 100 nucleotides, or a polynucleotide of at least 200 nucleotides. The antisense nucleic acids may be much longer constructs. The nucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The nucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, and may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, e.g., Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, 86:6553-6; Lemaitre *et al.*, *Proc. Natl. Acad. Sci. USA* 1987, 84:648-52; PCT Publication No. WO 88/09810) or blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134), hybridization triggered cleavage agents (see, e.g., Krol *et al.*, *BioTechniques* 1988, 6:958-76) or intercalating agents (see, e.g., Zon, *Pharm. Res.* 1988, 5:539-49).

In one embodiment disclosed herein, a VIAF antisense polynucleotide (including oligonucleotides) is provided, for example of single-stranded DNA. The VIAF antisense polynucleotide may recognize any species of VIAF. The antisense polynucleotide may be modified at any position on its structure with substituents generally known in the art. For example, a modified base moiety may be 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N˜6-sopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, methoxyarninomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-S-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

In another embodiment, the polynucleotide includes at least one modified sugar moiety such as arabinose, 2-fluoroarabinose, xylose, and hexose, or a modified component of the phosphate backbone, such as phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, or a formacetal or analog thereof.

In yet another embodiment, the polynucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.* 1987, 15:6625-41). The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent. Oligonucleotides may include a targeting moiety that enhances uptake of the molecule by tumor cells. The targeting moiety may be a specific binding molecule, such as an antibody or fragment thereof that recognizes a molecule present on the surface of the tumor cell.

As an alternative to antisense inhibitors, catalytic nucleic acid compounds, such as ribozymes or anti-sense conjugates, may be used to inhibit gene expression. Ribozymes may be synthesized and administered to the subject, or may be encoded on an expression vector, from which the ribozyme is synthesized in the targeted cell (as in PCT publication WO 9523225, and Beigelman *et al. Nucl. Acids Res.* 1995, 23:4434-42). Examples of oligonucleotides with catalytic activity are described in WO 9506764. Conjugates of antisense with a metal complex, e.g. terpyridylCu (II), capable of mediating mRNA hydrolysis, are described in Bashkin *et al.*, *Appl. Biochem Biotechnol.* 1995, 54:43-56.

Polynucleotides disclosed herein can be synthesized by standard methods known in the art, for example by use of an automated DNA synthesizer (Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligos may be synthesized by the method of Stein et al. (*Nucl. Acids Res.* 1998, 16:3209), methylphosphonate oligos can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:7448-51). In a specific embodiment, the VIAF antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see PCT International Publication WO 90/11364, Sarver et al., *Science* 1990, 247:1222-5). In another embodiment, the oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., *Nucl. Acids Res.* 1987, 15:6131-48), or a chimeric RNA-DNA analogue (Inoue et al., *FEBS Lett.* 1987, 215:327-330).

The antisense polynucleic acids disclosed herein comprise a sequence complementary to at least a portion of an RNA transcript of a *VIAF* gene, such as a human *VIAF* gene. However, absolute complementarity, although advantageous, is not required. A sequence may be complementary to at least a portion of an RNA, meaning a sequence having sufficient complementarily to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded VIAF antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a VIAF RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable

degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The relative ability of polynucleotides (such as oligonucleotides) to bind to complementary strands is compared by determining the melting temperature (T_m) of a hybridization complex of the poly/oligonucleotide and its complementary strand. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). A reduction in UV absorption indicates a higher T_m. The higher the T_m the greater the strength of the binding of the hybridized strands. As close to optimal fidelity of base pairing as possible achieves optimal hybridization of a poly/oligonucleotide to its target RNA.

The amount of VIAF antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In one embodiment, pharmaceutical compositions comprising VIAF antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In other embodiments, it may be useful to use such compositions to achieve sustained release of the VIAF antisense nucleic acids. In yet another embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti *et al. Proc. Natl. Acad. Sci. USA* 1990, 87:2448-51; Renneisen *et al. J. Biol. Chem.* 1990, 265:16337-42).

EXAMPLE 26

Methods of Treatment using Antisense Molecules

When VIAF levels are prematurely downregulated by various antisense strategies, the cells maybe induced into entering an apoptotic pathway. VIAF antisense oligonucleotides (EXAMPLE 25) can be used to disrupt cellular expression of a VIAF protein.

The subject suffering from a disease in which apoptosis is desired, can be treated with a therapeutically effective amount of VIAF antisense. After the VIAF antisense has taken affect (VIAF levels are downregulated), after 24-48 hours, the subject can be monitored for decreased apoptosis.

Prophylactic Treatments

The treatments disclosed herein can also be used prophylactially, for example to inhibit or prevent progression to of a disease in which apoptosis is is not desired. Such administration is indicated where the treatment is shown to have utility for treatment or prevention of the disorder. The prophylactic use is indicated in conditions known or suspected of preceding progression to diseases associated with an undesired amount of apoptosis, for example in diseases associated with VIAF expression. Such diseases may include cancer, heterocellular hereditary persistence of fetal

hemoglobin, deafness, cardiomyopathy, 6-12 leukemia, MALT lymphoma, and insulin dependent diabetes.

EXAMPLE 27

Cloning of VIAF Genomic DNA

Methods for cloning VIAF genomic DNA from any species are known to those skilled in the art, and are described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. 1989. Herein incorporated by reference). Briefly, VIAF cDNA (full length or fragments thereof, for example SEQ ID NOs 1, 3, 5, 7, and 9) is radiolabeled as described in EXAMPLE 4 with Rediprime II (Amersham Pharmacia Biotech) as instructed by the manufacturer. The radiolabeled cDNA is used to screen a bacteriophage lamda gt11 genomic library. Genomic DNA of the resulting positive clones is isolated, purified and digested with appropriate restriction enzymes. Digested DNA is separated by agarose gel electrophoresis and blotted onto a nylon membrane. A Southern-Blot is performed using radioactive cDNA of VIAF to identify the exons. Bands that hybridized with the cDNA are isolated from the gel and sequenced. The resulting DNA sequence is analyzed by specific computer programs to identify the promoter region and exon/intron donor/acceptor sites.

EXAMPLE 28

VIAF Transgenic Plants and Animals

The creation of transgenic plants and animals which express VIAF can be made by techniques known in the art, for example those disclosed in U.S. Patent Nos. 5,574,206; 5,723,719; 5,175,383; 5,824,838; 5,811,633; 5,620,881; and 5,767,337, which are incorporated by reference.

Methods for generating transgenic mice are described in *Gene Targeting*, Joyuner ed., Oxford University Press, 1995 and Watson *et al.*, *Recombinant DNA 2nd Ed.*, W.H. Freeman and Co., New York, 1992, Chapter 14. To generate transgenic mice containing a functional deletion of the VIAF gene, genomic fragments can be used as short arm and long arm. Between long arm and short arm, the *neo* gene is introduced, generating a the knock-out vector.

Using standard transgenic mouse technology, the knock-out vector can be used to generate VIAF knock-out mice by homologous recombination. The knock-out vector is introduced into embryonic stem cells (ES cells) by standard methods which may include transfection, retroviral infection or electroporation (also see EXAMPLE 14). Transfected ES cells expressing the knock-out vector will grow in medium containing the antibiotic G418. The neomycin resistant ES cells are microinjected into mouse embryos (blastocysts), which are implanted into the uterus of pseudopregnant mice. The resulting litter is screened for chimeric mice by observing their coat color. Chimeric mice are ones in which the injected ES cells developed into the germ line, thereby

allowing transmission of the gene to their offspring. The resulting heterozygotic mice will be mated to generate a homozygous line of transgenic mice functionally deleted for VIAF. These homozygous mice will then be screened phenotypically, for example, their predisposition to developing diseases which can include: cancer, heterocellular hereditary persistence of fetal hemoglobin, deafness, cardiomyopathy, 6-12 leukemia, MALT lymphoma, diabetes mellitus, and insulin dependent diabetes. Knock-out mice which do not express VIAF in their cells can be prepared to further investigate the role of VIAF on apoptosis pathways.

EXAMPLE 30

Generation and Expression of VIAF Fusion Proteins

Methods for making fusion proteins are well known to those skilled in the art. For example U.S. Patent No. 6,057,133 to Bauer *et al.* (herein incorporated by reference) discloses methods for making fusion molecules composed of human interleukin-3 (hIL-3) variant or mutant proteins functionally joined to a second colony stimulating factor, cytokine, lymphokine, interleukin, hematopoietic growth factor or IL-3 variant. U.S. Patent No. 6,072,041 to Davis *et al.* (herein incorporated by reference) discloses the generation of fusion proteins comprising a single chain Fv molecule directed against a transcytotic receptor covalently linked to a therapeutic protein.

Similar methods can be used to generate fusion proteins comprising VIAF (or variants or fragments thereof) linked to other amino acid sequences. Linker regions can be used to space the two portions of the protein from each other and to provide flexibility between them. The linker region is generally a polypeptide of between 1 and 500 amino acids in length, for example less than 30 amino acid in length. The linker joining the two molecules can be designed to (1) allow the two molecules to fold and act independently of each other, (2) not have a propensity for developing an ordered secondary structure which could interfere with the functional domains of the two proteins, (3) have minimal hydrophobic or charged characteristic which could interact with the functional protein domains and (4) provide steric separation of the two regions. Typically surface amino acids in flexible protein regions include Gly, Asn and Ser. Other neutral amino acids, such as Thr and Ala, can also be used in the linker sequence. Additional amino acids may also be included in the linker due to the addition of unique restriction sites in the linker sequence to facilitate construction of the fusions. Other moieties may also be included, as desired. These may include a binding region, such as avidin or an epitope, such as a polyhistadine tag, which may be useful for purification and processing of the fusion protein. In addition, detectable markers can be attached to the fusion protein, so that the traffic of the fusion protein through a body or cell may be monitored conveniently. Such markers may include radionuclides, enzymes, fluors, and the like.

Fusing of the nucleic acid sequences of VIAF (or variant or fragment thereof), with the nucleic acid sequence of another protein (or variant or fragment thereof), can be accomplished by

the use of intermediate vectors. Alternatively, one gene can be cloned directly into a vector containing the other gene. Linkers and adapters can be used for joining the nucleic acid sequences, as well as replacing lost sequences, where a restriction site was internal to the region of interest. Genetic material (DNA) encoding one polypeptide, peptide linker, and the other polypeptide is inserted into a suitable expression vector which is used to transform prokaryotic or eukaryotic cells, for example bacteria, yeast, insect cells or mammalian cells (see EXAMPLE 14). The transformed organism is grown and the protein isolated by standard techniques, for example by using a detectable marker such as nickel-chelate affinity chromatography, if a polyhistadine tag is used. The resulting product is therefore a new protein, a fusion protein, which has a VIAF joined by a linker region to a second protein. To confirm that the fusion protein was expressed, the purified protein is subjected to electrophoresis in SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane filters using established methods. The protein products can be identified by Western blot analysis using antibodies directed against the individual components, i.e., polyhistadine tag and VIAF (see EXAMPLES 5 and 15).

Having illustrated and described the principles of isolating several VIAF cDNAs, the proteins they encode, antibodies which recognize the proteins and modes of use of these biological molecules to modulate the anti-apoptotic and signaling properties of IAP family members, such as XIAP, it should be apparent to one skilled in the art that the disclosure can be modified in arrangement and detail without departing from such principles. In view of the many possible embodiments to which the principles of our disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. Rather, the scope of the disclosure is in accord with the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A purified or synthetic protein having VIAF biological activity, and comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 2, 4, 6 or 8;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

2. The protein of claim 1, wherein the amino acid sequence comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 2;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence s of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

3. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 4;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

4. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 6;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

5. The protein of claim 1, wherein the amino acid sequence comprises a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO 8;

(b) amino acid sequences that differ from those specified in (a) by one or more conservative amino acid substitutions that retain VIAF biological activity;

(c) fragments of the amino acid sequence of (a) or (b) that retain VIAF biological activity; and

(d) amino acid sequences having at least 80% sequence identity to the sequences specified in (a), (b) and (c) that retain VIAF biological activity.

6. The protein of claim 1(d), wherein the amino acid sequences have at least 90% sequence identity to the sequence specified in 1(a).

7. The protein of claim 1(d), wherein the amino acid sequences have at least 95% sequence identity to the sequence specified in 1(a).

8. The protein of claim 1(d), wherein the amino acid sequences have at least 98% sequence identity to the sequence specified in 1(a).

9. The protein of claim 1, wherein the amino acid sequence contains no more than 270 amino acid residues.

10. The protein of claim 1, wherein the amino acid sequence contains no more than 260 amino acid residues.

11. The protein of claim 1, wherein the amino acid sequence contains no more than 250 amino acid residues.

12. An animal protein having VIAF biological activity.

13. The animal protein of claim 12, wherein the animal is a mammal.

14. The animal protein of claim 12, wherein the mammal is a human.

15. An isolated nucleic acid molecule encoding a protein according to any of claims 1-10.

16. The isolated nucleic acid of claim 15, further comprising a promoter sequence operably linked to the nucleic acid of claim 15.

17. An isolated nucleic acid molecule, wherein the nucleic acid molecule includes the sequence selected from the group consisting of:

(a) SEQ ID NO 1 or its complementary strand;

(b) SEQ ID NO 3 or its complementary strand;

(c) SEQ ID NO 5 or its complementary strand;

(d) SEQ ID NO 7 or its complementary strand; and

(e) Sequences which hybridize under conditions of at least 75% stringency to the sequences defined in (a), (b), (c), or (d).

18. The isolated nucleic acid molecule of claim 17, wherein the nucleic acid comprises the nucleic acid sequence depicted as nucleotides 62-781 of SEQ ID NO 1.

19. The isolated nucleic acid molecule of claim 17, wherein the nucleic acid includes a sequence selected from the group consisting of:

(a) at least 370 contiguous nucleotides of SEQ ID NO 1 or its complementary strand;

(b) at least 20 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(c) at least 30 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(d) at least 50 contiguous nucleotides from nucleotides 62-334, 1-334, or 703-781 of SEQ ID NO 1, or its complementary strand;

(e) at least 21 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(f) at least 30 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(g) at least 50 contiguous nucleotides of SEQ ID NO 3 or its complementary strand;

(h) at least 22 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(i) at least 30 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(j) at least 50 contiguous nucleotides of SEQ ID NO 5 or its complementary strand;

(k) at least 21 contiguous nucleotides of SEQ ID NO 7 or its complementary strand;

(l) at least 30 contiguous nucleotides of SEQ ID NO 7 or its complementary strand; and

(m) at least 50 contiguous nucleotides of SEQ ID NO 7 or its complementary strand.

20. An isolated nucleic acid molecule that:

(a) is at least 80% homologous to SEQ ID NOS 1, 3, 5, or 7; and

(b) encodes a protein having VIAF biological activity.

21. A recombinant vector including a nucleic acid molecule according to any of claims 15-20.

22. A transgenic cell produced by introducing into a cell a recombinant vector according to claim 21.

23. A purified protein encoded by the nucleic acid molecule according to any of claims 15-20.

24. The protein of claim 23, wherein the protein has an amino acid sequence as shown in SEQ ID NO 2, 4, 6, or 8.

25. The isolated nucleic acid molecule of any of claims 15-20 wherein the nucleic acid molecule encodes a peptide having VIAF biological activity.

26. The isolated nucleic acid molecule according to claim 17(e) wherein the nucleic acid molecule hybridizes under conditions of at least 90% stringency to the sequences defined in claim 17 (a), claim 17 (b), claim 17(c), or claim 17 (d).

27. An antisense oligonucleotide which:

hybridizes to an RNA or a plus strand of a nucleic acid any of claims 15-20; and

inhibits VIAF biological activity.

28. A specific binding agent capable of specifically binding to a VIAF protein.

29. The specific binding agent of claim 28 wherein the specific binding agent is selected from the group consisting of: polyclonal antibodies; monoclonal antibodies; and fragments of monoclonal antibodies.

30. A composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of the protein of any of claims 1-14 or 23-24.

31. The composition of claim 30, further comprising one or more other anti-apoptotic compounds.

32. A composition comprising a therapeutically effective amount of the specific binding agent of claim 28, and a pharmaceutically acceptable carrier.

33. The compositions of any of claims 30-32, for use in decreasing apoptosis.

34. The composition of claim 33, for use in a subject suffering from unwanted apoptosis, in an amount sufficient to inhibit Bax- and Fas-induced apoptosis in the subject.

35. The composition of claim 34, wherein the subject has a cancer, an autoimmune disease, or a neurodegenerative disease characterized by unwanted apoptosis.

36. A composition comprising a therapeutically effective amount of the antisense oligonucleotide of claim 27, and a pharmaceutically acceptable carrier.

37. The composition of claim 36, for use in a subject with a disease, in whom apoptosis is desired.

38. A method for detecting an enhanced susceptibility of a subject to a disease of abnormal apoptosis, the method comprising detecting a deletion of or within a VIAF gene in cells of the subject, or detecting a decrease or absence of VIAF protein in cells of the subject.

39. The method of claim 38 wherein the disease is selected from the group consisting of: cancers, autoimmune diseases; and neurodegenerative diseases.

40. The method of claim 38, wherein the method comprises detecting a deletion of or within a VIAF gene in a cell, the method comprising:

incubating the nucleic acid of claim 17 with a nucleic acid of the cell under conditions such that the nucleic acid will specifically hybridize to the VIAF gene present in the nucleic acid to form an nucleic acid:VIAF gene complex;

detecting an increase or decrease of the nucleic acid:VIAF complexes, wherein an absence of the complexes indicates deletion of or within the VIAF gene.

41. The method of claim 38, wherien the method comprises:

incubating the specific binding agent of claim 28 with proteins of the cell under conditions such that the specific binding agent will specifically bind to a VIAF protein present in the cell to form a specific binding agent:VIAF protein complex; and

detecting an increase or decrease of specific binding agent:VIAF protein complexes.

42. A method of treating a disease of abnormal apoptosis by supplying VIAF biological activity to a cell which has lost the VIAF biological activity by a deletion of all or a portion of a VIAF gene, comprising introducing the nucleic acid of claim 10 into the cell such that the nucleic acid of claim 17 is expressed in the cell.

43. A method of decreasing apoptosis in a cell comprising increasing the level of VIAF biological activity, which prevents the cell from undergoing apoptosis.

44. The method of claim 43, whereby decreasing apoptosis inhibits Bax- and Fas-induced apoptosis.

45. The method of claim 44, wherein the cell is characterized by:

(a) decreased expression of a VIAF protein; or

(b) decreased VIAF biological activity or expression, relative to VIAF biological activity or expression in a same tissue type that is undergoing apoptosis.

46. The method of claim 45, wherein increasing the level of VIAF biological activity comprises exposing the cell to a therapeutically effective amount of the VIAF protein of claim 1.

47. The method of claim 46, wherein increasing the level of VIAF biological activity comprises administering a therapeutically effective amount of a nucleic acid which can express a protein having VIAF biological activity.

48. The method of claim 47, wherein the nucleic acid is the nucleic acid of claim 13.

49. The method of claim 43 or the composition of claim 33, wherein decreasing apoptosis treats a disease caused by defects in apoptosis.

50. The method of claim 49 or the composition of claim 34 wherein the disease is selected from the group consisting of autoimmune and neurodegenerative diseases

51. The method and composition of claim 50, wherein the disease is selected from the group consisting f: diabetes, multiple sclerosis and retinal degeneration.

52. A method for inducing apoptosis by adding a therapeutically effective amount of the antisense oligonucleotide of claim 27 sufficient to induce apoptosis in a subject.

53. The method of claim 52 and the composition of claim 37, wherein apoptosis is induced to treat a disease caused by defects in apoptosis.

54. The method and composition of claim 53, wherein the disease is a cancer.

55. A method of treating a disease caused by a mutation in the nucleic acid of claim 17 by supplying therapeutically effective amounts of a polypeptide product or the nucleic acid.

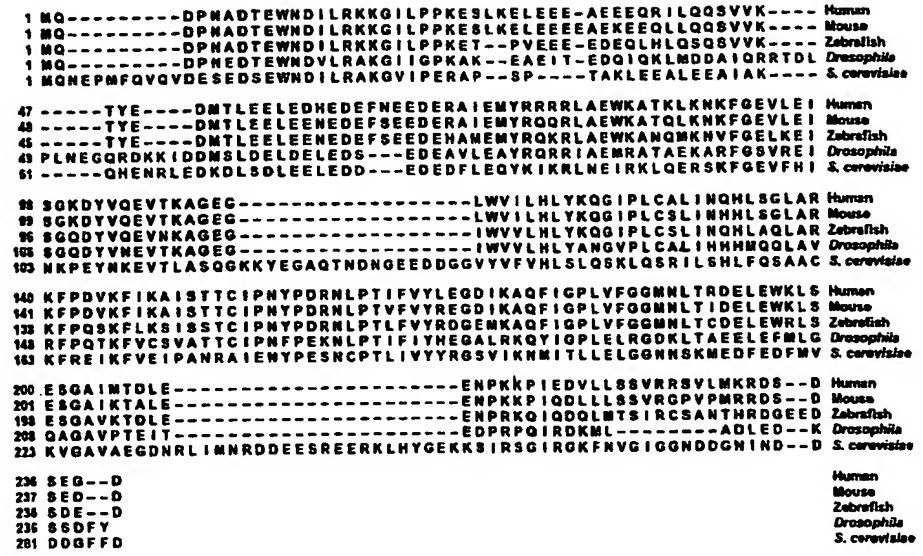
56. The protein of claim 1, for use in modulating apoptosis.

57. The nucleic acid of claim 15, for use in modulating apoptosis.

58. The protein of claim 1, for use in inhibiting apoptosis.

59. The antisense oligonucleotide of claim 27, for use in increasing apoptosis.

FIG. 1



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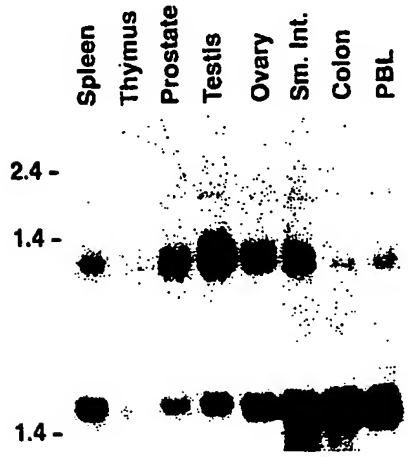

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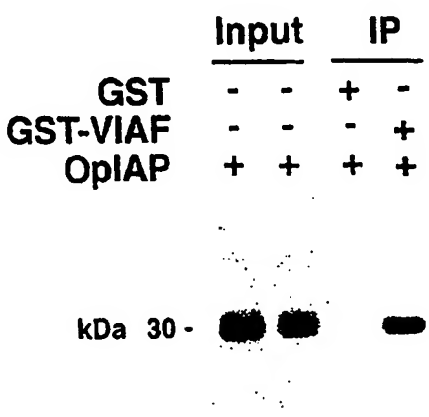

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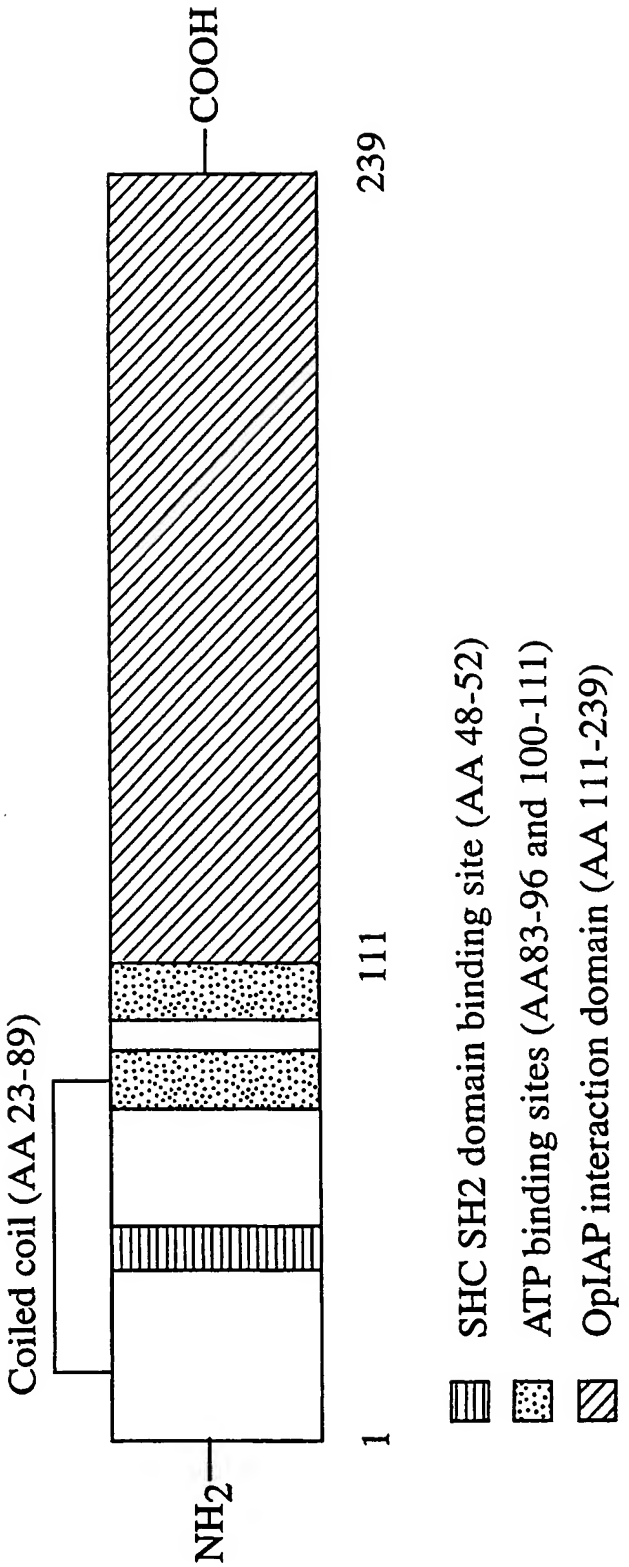
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ATP binding sites (AA83-96 and 100-111)
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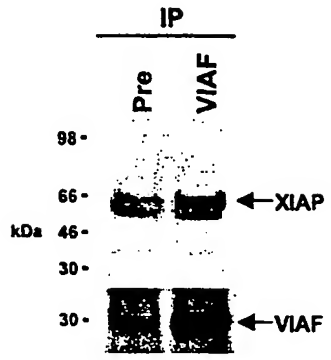
FIG. 5



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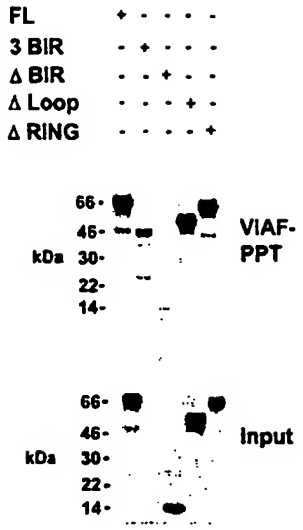


FIG. 7

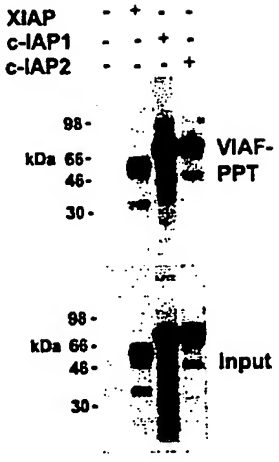
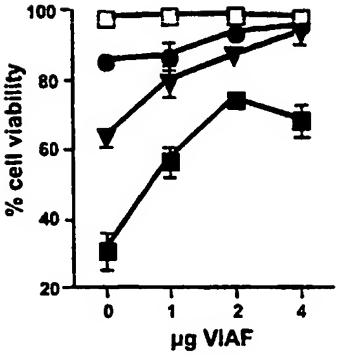


FIG. 8A



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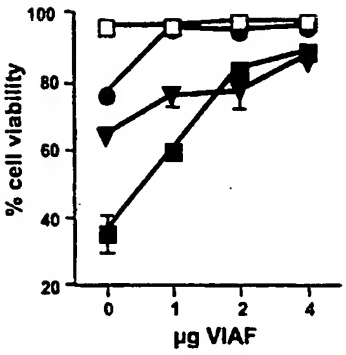


FIG. 9

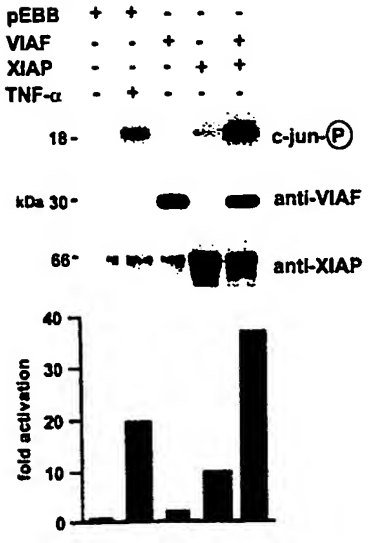
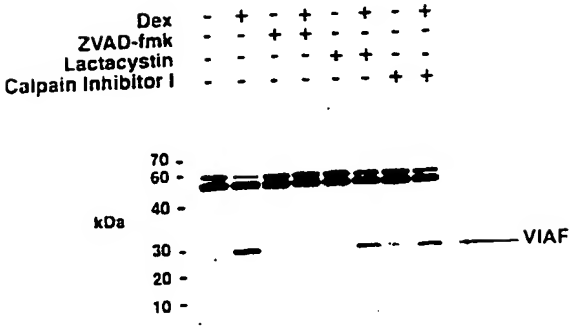


FIG. 10



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Lys Gly Ile Ile Gly Pro Lys Ala Lys Glu Ala Glu Ile Thr Glu Asp
             20                  25                  30

cag atc cag aag ctg atg gac gat gcc atc cag cgg cgc aca gat ctg   144
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gcg cga ttt gga tca gtg cgc gag atc tca gga cag gat tat gtc aac   336
Ala Arg Phe Gly Ser Val Arg Glu Ile Ser Gly Gln Asp Tyr Val Asn
            100                 105                 110

gag gtg acc aag gcc ggc gag ggc atc tgg gtg gta ctc cac ctg tat   384
Glu Val Thr Lys Ala Gly Glu Gly Ile Trp Val Val Leu His Leu Tyr
        115                 120                 125

gcc aac ggc gta ccg ctg tgc gca ctg atc cac cat cac atg cag cag   432
Ala Asn Gly Val Pro Leu Cys Ala Leu Ile His His His Met Gln Gln
    130                 135                 140

ctg gcc gtc cgc ttt cca cag acc aag ttc gtg tgc tcc gtt gcc acc   480
Leu Ala Val Arg Phe Pro Gln Thr Lys Phe Val Cys Ser Val Ala Thr
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acc tgc ata cca aac ttc ccc gag aag aac ctg ccc acc atc ttt atc   528
Thr Cys Ile Pro Asn Phe Pro Glu Lys Asn Leu Pro Thr Ile Phe Ile
                165                 170                 175

tac cac gag ggt gcg ctg cgc aag cag tac ata ggc cca ctg gag ctg   576
Tyr His Glu Gly Ala Leu Arg Lys Gln Tyr Ile Gly Pro Leu Glu Leu
            180                 185                 190

cgc ggc gac aag ttg acc gct gag gag ctg gag ttt atg ctg ggt cag   624
Arg Gly Asp Lys Leu Thr Ala Glu Glu Leu Glu Phe Met Leu Gly Gln
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gcg gga gca gtg ccc acc gag atc acg gag gat cca cgg ccg cag atc   672
Ala Gly Ala Val Pro Thr Glu Ile Thr Glu Asp Pro Arg Pro Gln Ile
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Gln Ile Gln Lys Leu Met Asp Asp Ala Ile Gln Arg Arg Thr Asp Leu
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Pro Leu Asn Glu Gly Gln Arg Asp Lys Lys Ile Asp Asp Met Ser Leu
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Asp Glu Leu Asp Glu Leu Glu Asp Ser Glu Asp Glu Ala Val Leu Glu
65                  70                  75                  80
Gln Tyr Arg Gln Arg Arg Ile Ala Glu Met Arg Ala Thr Ala Glu Lys
                85                  90                  95
Ala Arg Phe Gly Ser Val Arg Glu Ile Ser Gly Gln Asp Tyr Val Asn
            100                 105                 110
Glu Val Thr Lys Ala Gly Glu Gly Ile Trp Val Val Leu His Leu Tyr
        115                 120                 125
Ala Asn Gly Val Pro Leu Cys Ala Leu Ile His His His Met Gln Gln
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Leu Ala Val Arg Phe Pro Gln Thr Lys Phe Val Cys Ser Val Ala Thr
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Thr Cys Ile Pro Asn Phe Pro Glu Lys Asn Leu Pro Thr Ile Phe Ile
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Tyr His Glu Gly Ala Leu Arg Lys Gln Tyr Ile Gly Pro Leu Glu Leu
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Arg Gly Asp Lys Leu Thr Ala Glu Glu Leu Glu Phe Met Leu Gly Gln
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gca cct tcg ccc act gca aag tta gaa gaa gca tta gaa gaa gca att   144
Ala Pro Ser Pro Thr Ala Lys Leu Glu Glu Ala Leu Glu Glu Ala Ile
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Ala Lys Gln His Glu Asn Arg Leu Glu Asp Lys Asp Leu Ser Asp Leu
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gaa gaa cta gaa gac gat gaa gat gaa gat ttc ttg gaa gct tac aag   240
Glu Glu Leu Glu Asp Asp Glu Asp Glu Asp Phe Leu Glu Ala Tyr Lys
65                  70                  75                  80

atc aaa aga tta aat gaa atc cgc aaa tta cag gaa cgt tcc aaa ttt   288
Ile Lys Arg Leu Asn Glu Ile Arg Lys Leu Gln Glu Arg Ser Lys Phe
                85                  90                  95

gga gaa gtt ttc cac att aac aaa cct gaa tac aac aaa gag gtt act   336
Gly Glu Val Phe His Ile Asn Lys Pro Glu Tyr Asn Lys Glu Val Thr
            100                 105                 110

ttg gcc agt cag gga aag aaa tat gaa ggt gca caa acc aat gac aat   384
Leu Ala Ser Gln Gly Lys Lys Tyr Glu Gly Ala Gln Thr Asn Asp Asn
        115                 120                 125

ggt gaa gag gat gac ggt ggt gtc tac gta ttc gtt cat ctc tcg ctt   432
Gly Glu Glu Asp Asp Gly Gly Val Tyr Val Phe Val His Leu Ser Leu
    130                 135                 140

caa agt aaa cta caa agc aga att ctg tct cat ctt ttc caa tct gct   480
Gln Ser Lys Leu Gln Ser Arg Ile Leu Ser His Leu Phe Gln Ser Ala
145                 150                 155                 160

gca tgc aaa ttc aga gaa ata aaa ttt gta gaa ata cct gcc aat aga   528
Ala Cys Lys Phe Arg Glu Ile Lys Phe Val Glu Ile Pro Ala Asn Arg
                165                 170                 175

gca att gaa aac tat ccc gaa tcc aat tgc ccg aca tta att gta tat   576
Ala Ile Glu Asn Tyr Pro Glu Ser Asn Cys Pro Thr Leu Ile Val Tyr
            180                 185                 190

tac cgg ggt gag gta atc aaa aac atg ata acg cta cta gaa ctg ggt   624
Tyr Arg Gly Glu Val Ile Lys Asn Met Ile Thr Leu Leu Glu Leu Gly
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ggt aat aat tcc aag atg gaa gac ttt gaa gat ttt atg gta aaa gtt   672
Gly Asn Asn Ser Lys Met Glu Asp Phe Glu Asp Phe Met Val Lys Val
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ggc gct gtt gca gaa gga gac aac aga ctg ata atg aac cga gac gat   720
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Ala Lys Gln His Glu Asn Arg Leu Glu Asp Lys Asp Leu Ser Asp Leu
     50                  55                  60
Glu Glu Leu Glu Asp Asp Glu Asp Glu Asp Phe Leu Glu Ala Tyr Lys
 65                  70                  75                  80
Ile Lys Arg Leu Asn Glu Ile Arg Lys Leu Gln Glu Arg Ser Lys Phe
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Gly Glu Val Phe His Ile Asn Lys Pro Glu Tyr Asn Lys Glu Val Thr
            100                 105                 110
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        115                 120                 125
Gly Glu Glu Asp Asp Gly Gly Val Tyr Val Phe Val His Leu Ser Leu
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Gln Ser Lys Leu Gln Ser Arg Ile Leu Ser His Leu Phe Gln Ser Ala
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Ala Cys Lys Phe Arg Glu Ile Lys Phe Val Glu Ile Pro Ala Asn Arg
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Ala Ile Glu Asn Tyr Pro Glu Ser Asn Cys Pro Thr Leu Ile Val Tyr
            180                 185                 190
Tyr Arg Gly Glu Val Ile Lys Asn Met Ile Thr Leu Leu Glu Leu Gly
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Gly Ala Val Ala Glu Gly Asp Asn Arg Leu Ile Met Asn Arg Asp Asp
225                 230                 235                 240
Glu Glu Ser Arg Glu Glu Arg Lys Leu His Tyr Gly Glu Lys Lys Ser
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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/20576

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/11 C07K14/47 C07K16/18 C12Q1/68
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, EMBL, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUZUKI ATSUSHI ET AL: "Resistance to Fas-mediated apoptosis: Activation of capase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP." ONCOGENE, vol. 17, no. 8, August 1998 (1998-08), pages 931-939, XP000979228 ISSN: 0950-9232 abstract --- -/--	12-14

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2001	14/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/20576

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! 28 January 1998 (1998-01-28) MARRA M., ET AL.: "similar to TR:Q12017 Q12017 HYPOTHETICAL 32.8 KD PROTEIN." retrieved from EBI Database accession no. AA763356 XP002158477 abstract	1,3, 6-13, 15-17, 19, 21-27, 29-41, 56-59
X	DATABASE EMBL 'Online! 1 February 1999 (1999-02-01) HARVEY D. ET AL.: "Drosophila melanogaster cDNA clone GH20282 5prime, mRNA sequence." retrieved from EBI Database accession no. AI389174 XP002158481 abstract	1,5-12, 15-17, 19, 21-27, 29-41, 56-59
A	DATABASE EMBL 'Online! 7 January 1999 (1999-01-07) MATTHEWS L.: "Human DNA sequence from clone 44A20 on chromosome 6q23.1-24.3" retrieved from EBI Database accession no. AL035086 XP002158478 abstract	2,6-13, 15-18, 21-27, 29-41, 56-59
A	DATABASE TREMBLREL 'Online! 1 November 1999 (1999-11-01) WILSBACHER L.D. ET AL.: "The mouse Clock locus: Sequence and analysis of 204 kb from mouse . TPHLP (FRAGMENT)" retrieved from EBI Database accession no. Q9WUP3 XP002158479 abstract	1-27, 29-41, 56-59
A	DATABASE EMBL 'Online! 16 March 1999 (1999-03-16) CLARK M. ET AL.: "Q12017 HYPOTHETICAL 32.8 KD PROTEIN. ; mRNA sequence." retrieved from EBI Database accession no. AI437036 XP002158480 abstract	1-27, 29-41, 56-59

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